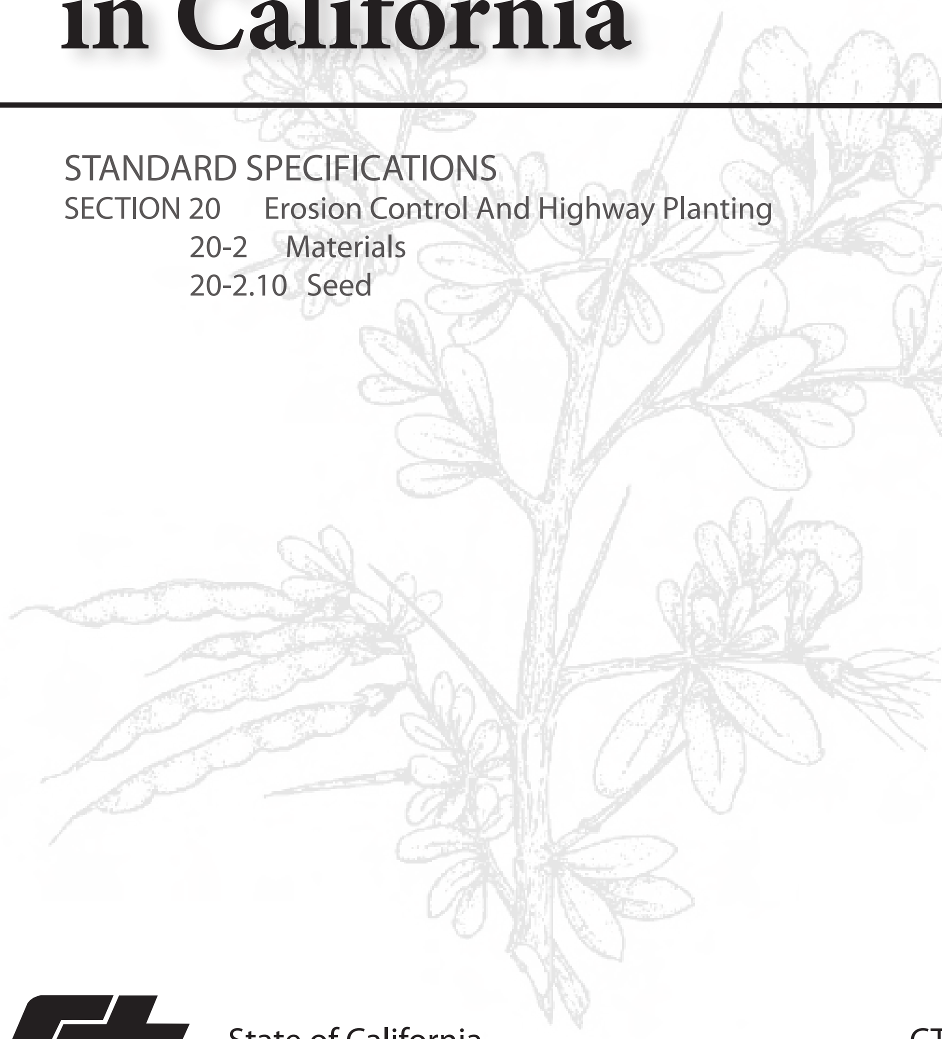
Roadside Erosion Control and Management Reviews

A Review of

Legume Seed Inoculation for Highway Planting in California



STANDARD SPECIFICATIONS
SECTION 20 Erosion Control And Highway Planting
20-2 Materials
20-2.10 Seed

Final

State of California
Department of Transportation

CTSW-RT-06-167.01.2
July 2006

Acknowledgments
This review was complied as part of the Roadside Erosion Control and Management Study, a multi-year cooperative project among:

- Caltrans, Division of Design
- Sacramento State University Office of Water Programs, and the
- Earth and Soil Sciences Department of Cal Poly State University, San Luis Obispo.

Linda Allen, Research Associate, Earth and Soil Sciences Department, Cal Poly State University, San Luis Obispo, authored this report, and conducted the literature searches and reviews.

Michael Curto, Senior Research Associate, Earth and Soil Sciences Department, Cal Poly State University, San Luis Obispo, edited and formatted the document.

Dennis Cadd, Senior Landscape Architect, Headquarters, requested this review as Task Manager for the Roadside Erosion Control and Management Study, and offered comments on a draft.

Brent Hallock, Professor, Earth and Soil Sciences Department, Cal Poly State University, San Luis Obispo, and Principal Investigator for the Roadside Erosion Control and Management Study, facilitated production.

Cover illustration: Chaparral Pea, *Pickeringia montana* (Fabaceae/Leguminosae) from

Sampson, AW; Jespersen, BS. 1963. California Range and Browse Plants. *University of California Experiemnt Station Bulletin* **4010**.

Contents

List of Tables ... ii
List of Figures ... ii

Executive Summary ... ES-1

1.0 Introduction ... 1-1
1.1 Task Objectives ... 1-1
1.2 Tasks ... 1-1
1.3 Scope of Review ... 1-2
1.4 Limits of Applicability ... 1-2
1.5 Bacterial Taxonomy ... 1-2
1.6 Review Format ... 1-3

2.0 Standard Specification 20-2.10 ... 2-1

3.0 Summary Review of Standard Specification 20-2.10 ... 3-1
3.1 Intended Usage of Bulletin 1842 Protocol ... 3-1
3.2 Inoculation Provider ... 3-2
3.3 Selection of Rhizobial Strains ... 3-3
3.4 Inoculant Purity ... 3-5
3.5 Inoculant Shelf Life and Storage Conditions ... 3-6
3.6 Rate of Inoculation ... 3-8
3.7 Post-Inoculation Seed Storage ... 3-10
3.8 Inoculated Seed Application Method ... 3-11
3.9 Inoculated Seed Application Rate ... 3-12
3.10 Site Physical Conditions ... 3-13

4.0 Expanded Review of Standard Specification 20-2.10 ... 4-1
4.1 Intended Usage of Bulletin 1842 Protocol ... 4-1
4.2 Inoculation Provider ... 4-16
4.3 Selection of Rhizobial Strains ... 4-17
4.4 Inoculant Purity ... 4-21
4.5 Inoculant Shelf Life and Storage Conditions ... 4-23
4.6 Rate of Inoculation ... 4-25
4.7 Post-Inoculation Seed Storage ... 4-28
4.8 Inoculated Seed Application Method ... 4-30
4.9 Inoculated Seed Application Rate ... 4-32
4.10 Site Physical Conditions ... 4-34

5.0 Actions & Recommendations ... 5-1
5.1 Action Alternatives ... 5-1

6.0 References ... 6-1

Appendix A Glossary ... A-1

Contents

List of Tables

Table 3-1. Comparison of How Rangeland Protocol of *UC-AES Bulletin 1842* Applies to Roadsides 3-1

Table 3-2. Synopsis of Potential Problems With Inoculation Factors Relative to Provider Category 3-2

Table 3-3. Inoculation Factors: Summary of Problems and Specification Revision Options 3-16

Table 4-1. Comparison of How Rangeland Protocol of *UC-AES Bulletin 1842* Applies to Roadsides 4-1

Table 4-2. Major Forms of Atmospheric Nitrogen Deposition 4-5

Table 4-3. Relative Magnitude of Nitrogen Deposition 4-7

Table 4-4. Estimates of Relative N Inputs from Atmospheric Deposition & Biological Nitrogen Fixation (BNF) 4-8

Table 4-5. Diagnosis of the Need to Inoculate Roadside Revegetation 4-14

Table 4-6. Synopsis of Potential Problems With Inoculation Factors Relative to Provider Category 4-16

Table 4-7. Effects of Vetch Seed Immersion in Hydroseed Mix 4-30

Table 5-1. Summary of Present Control of Legume Inoculation Factors and Potential Modifications to Improve Efficacy 5-1

Table 5-2. Nitrogen Augmentation Methods For Unvegetated Roadside Soils 5-4

List of Figures

Figure 4-1. Seasonal Relationship Between Climate and N Dynamics in California Annual Grasslands 4-4

Figure 4-2. The Nitrogen Cycle 4-6

Figure 4-3. The Nitrogen Cycle 4-6

Figure 4-4. Well-Nodulated Root System 4-18

Figure 4-5. Rhizobial Inoculant Purity 4-21

Figure 4-6. Legume to Non-Legume Seed Rate Ratios (kg PLS/ha) in 30 Selected Caltrans Erosion Control Specifications 4-32

Executive Summary

For at least three decades, Caltrans has routinely incorporated into highway planting specifications a requirement for seeds of legume species to be inoculated with N_2-fixing bacteria as a means to augment soil nitrogen (N) levels on disturbed roadsides. As part of ongoing evaluations by Caltrans of standard erosion control and highway planting specifications, a review of the basic science and practice of legume seed inoculation is necessary 1) to ascertain whether documented evidence exists to indicate that legume inoculation for N-augmentation is both consistently reliable and quantifiable, and 2) to provide Caltrans with potential action alternatives regarding existing specifications.

The present California State Standard Specification 20-2.10 for legume seed inoculation derives from the *University of California Agricultural Experiment Station Bulletin 1842*, "Range-Legume Inoculation and Nitrogen Fixation by Root-Nodule Bacteria," published in 1987.

Ten primary factors affect efficacy of legume inoculation, and problems exist with how the present Caltrans legume inoculation protocol manages these factors.

1) Intended Usage of UC-AES Bulletin 1842 Protocol

Intended use of the legume inoculation protocol is by ***ranchers*** on ***rangeland pastures*** for ***livestock grazing***, not for revegetation of roadsides or wildlands.

2) Inoculation Provider

The inoculation process may not be equivalent among commercial/industrial professionals, seed vendors, or landscape contractors.

3) Selection of Rhizobial Strains

Lack of industry-wide standards regarding strain compatibility or effectiveness of N_2-fixation.

4) Inoculant Purity

Lack of industry-wide standards regarding purity, and a wide variance in purity among manufacturers.

5) Inoculant Shelf Life and Storage Conditions

There are no requirements to ensure that inoculant has been stored appropriately so that the bacteria meet a quantitative minimum standard of viability.

6) Rate of Inoculation

Lack of industry-wide standards regarding appropriate application rates, or effectiveness of inoculation. *UC-AES Bulletin 1842* provides no guidance regarding inoculation of California native legumes specified in roadside seed mixes.

7) Post-Inoculation Seed Storage

The 90-day post-inoculation storage period currently allowed by SS 20-2.10 is far too long. A high potential exists for near complete mortality of rhizobia caused by high temperatures and desiccation within the first four hours of post-inoculation storage.

8) Inoculated Seed Application Method

UC-AES Bulletin 1842 refers only to pelleted drill-seeding of rangelands. Hydroseeding has the potential to damage seed or inoculant through extended immersion in the hydroseeder tank solution, acidity of the solution caused by fertilizers, and physical dislodgement of inoculant from seed during agitation and application processes.

9) Inoculated Seed Application Rate

Standard Specification 20-2.10 does not specify final legume plant densities. *UC-AES Bulletin 1842* recommends legume density ≥ 20 plants per ft^2. Caltrans project seeding rates for legumes are highly variable, but typically do not exceed 3 plants per ft^2.

10) Site Physical Conditions

There are no specifications restricting the seasonal timing of seed application. *UC-AES Bulletin 1842* recommends that inoculated seed be planted into a moist seedbed that will receive a germinating rain soon thereafter. Seeding during the hot and dry months of late spring, summer, or early fall will likely kill all inoculant.

No requirements to quantify soil fertility or pH in order to ascertain the need for legume inoculation or chemical fertilization. No specifications regarding testing for indigenous rhizobia that may suppress inoculated rhizobia.

Landscape architects must make decisions regarding environmental factors in the absence of data.

ACTION ALTERNATIVES

Following are five Action Alternatives regarding Standard Specification 20-2.10. **Alternatives 2, 3, and 4 require increasing input of time and money.**

RECOMMENDED ALTERNATIVE

1 DISCONTINUE OR DE-EMPHASIZE CURRENT LEGUME INOCULATION PRACTICES

The existing practices make it unlikely that much, if any, N_2-fixation from cultured legume inoculants is occurring during roadside revegetation. The degree to which inoculated legume N_2-fixation has been effective on roadside revegetation projects remains undocumented.

Given the present problems listed previously, the recommendation of this review is that Caltrans abandon SS 22-2.10 or limit its use to special cases at the discretion of project designers.

Instead, recommended routine soil fertility testing, topsoil stockpiling, and other means for soil nitrogen augmentation, would likely provide greater long-term management benefits.

Soil Fertility Testing

Establish routine soil fertility testing of all soils scheduled for revegetation to provide data necessary for informed decisions about nutrient augmentations and plant material selections.

Explore practical options for soil nitrogen augmentation beyond legume seed inoculation, especially organic amendments and microbiota.

Topsoil Harvesting And Stockpiling

Explore practical options for stockpiling and reapplying topsoil or duff to post-construction roadsides as the most effective way to retain organic matter, nitrogen and nutrient cycling within the soil ecosystem.

Establish routine soil seedbank testing of all soils scheduled for revegetation to provide data about presence and abundance of plant species and whether addition of seed is necessary.

2 IMPLEMENT BASIC PROCEDURAL CHANGES TO LEGUME SEED STANDARD SPECIFICATIONS

Ascertain the quantitative need for inoculation. Require post-construction soil testing, especially fertility, for every project requiring revegetation.

If specifying legume inoculation:

- Define inoculation rates for all species of legumes used in seed mix;
- Shorten the time between legume seed inoculation and seed application;
- Define post-inoculation seed storage conditions: refrigeration and transport in cooler to site;
- Restrict the timing of roadside seed application to coincide with impending rainfall season;
- Dry broadcast legume seeds separately; do not add them into the hydroseed mixture;
- Do not fertilize with N;
- Augment other nutrients critical to N_2-fixation as needed.

Reformat and condense *UC-AES Bulletin 1842* in order to define and standardize the practice of legume-inoculation as a method of N-augmentation.

3 IMPLEMENT SIGNIFICANT PROCEDURAL CHANGES TO STANDARD SPECIFICATIONS

Implement Alternative 3 plus the following:

- Require an inoculant purity test and viability test for every project with legume inoculation;
- Require a seed inoculation test to quantify and confirm the average number of rhizobia per seed for each species of legume called for.

4 EFFECT RESEARCH REGARDING LEGUME INOCULATION ON CALTRANS ROADSIDES PRIOR TO CHANGING THE STANDARD SPECIFICATIONS

- Quantify the success of legume inoculation as currently executed.
- Quantify the effects of indigenous rhizobia.
- Quantify the amount of N input potential for all legume species used.
- Develop guidelines to correlate legume seeding rates with target N input levels.
- Quantify the persistence of legumes on-site over 5 consecutive years.
- Explore other N-augmentation alternatives.

5 NO CHANGE : Do not change legume seed standard specifications

Assumes that district level landscape architects can best ascertain:

1) the need for N-augmentation through the use of inoculated legumes;
2) appropriate project-specific modifications to the standard specifications or *UC-AES Bulletin 1842*.

Section 1
Introduction

Legumes are all members of the vascular plant family Leguminosae, also called Fabacae after the broadbean genus *Faba*. The legume family includes about 18,000 species worldwide, with about 325 native species in California.

Important genera in revegetation of California roadsides include:

Acacia	Acacia
Lathyrus	Vetch
Lotus	Lotus
Lupinus	Lupin
Trifolium	Clover

Over the last century, agricultural research conducted to promote greater yields from legume crops provided empirical evidence for a general recommendation that legume establishment from seed is enhanced by artificial inoculation of seeds with host-specific root-nodule bacteria, hereafter referred to as **rhizobia** (e.g., *Bradyrhizobium* spp., *Rhizobium* spp.), capable of fixing atmospheric nitrogen (N_2) into plant-usable forms as ammonium or nitrates. Inoculation is done to ensure that rhizobia will be in close proximity to primary roots as they emerge and grow.

For at least three decades, Caltrans has routinely incorporated into highway planting specifications a requirement for seeds of legume species to be inoculated with N_2-fixing bacteria as a means to augment soil nitrogen (N) levels on disturbed roadsides. As part of ongoing evaluations by Caltrans of standard erosion control and highway planting specifications, a review of the basic science and practice of legume seed inoculation is necessary.

1.1 Task Objective

- Conduct a literature review of current thinking on the effectiveness of inoculating legume seed with rhizobia to evaluate whether documented evidence exists to indicate that legume inoculation for N-augmentation is both consistently reliable and quantifiable.
- Provide Caltrans with potential action alternatives regarding existing specifications.

1.2 Tasks

- Devise a list of search terms pertaining to inoculation of legume seed with rhizobia.
- Conduct review of online and hardcopy journal articles, books, government documents, and other references.
- Write a synopsis of current thinking on the effectiveness of inoculating legume seed with rhizobia to evaluate whether Caltrans should still require inoculation of seed applied by contractors.

1.3 Scope of Review

Discussion and recommendations incorporate basic ecological literature about disturbance, succession, and nitrogen availability..

The issue of legume seed inoculation cannot be isolated from a general ecological context of acute soil disturbance, N availability, the rhizosphere, and successional models. Thus, cumulative findings from the more comprehensive fields of resource management, land reclamation, and restoration ecology were examined to provide general background for the focused topic of legume seed inoculation for roadside revegetation.

1.4 Limits of Applicability

This review addresses legume seed inoculation for typical post-construction revegetation projects only.

The scope-of-interest herein pertains to methods of establishing roadside vegetation subsequent to highway construction activity on appropriately engineered roadsides. This review is not intended to address roadside conditions with acute physical instability or other geological complications. Such cases require engineering solutions that go beyond the establishment of a thin veneer of vegetation intended to intercept precipitation and mitigate soil loss and hydrologic runoff. Of the 13 factors that contribute to soil loss along Caltrans roadsides [*fide* **34**], the 8 which fall into the engineering and maintenance categories cannot be addressed in this review. These include steep slopes, inappropriate/non-functional site design, inadequate drainage systems, runon from adjacent watershed areas, malfunctioning irrigation systems, adverse physical properties of soils, improper site maintenance, and improper installation and management of physical erosion control devices.

1.5 Bacterial Taxonomy

Classification and taxonomy of the legume rhizobial bacteria is undergoing a vigorous revision based on molecular methods. The integrity of the traditional cross-inoculation group classification system has been brought into question and is now in general disrepute. The literature reviewed spans a broad timeframe; thus, the nomenclature used herein will conform to the publication being cited as it is beyond the scope of this review to provide a crosswalk among presently unresolved classification schema.

1.6 Review Format

This review is oriented toward current Caltrans objectives, expectations and methods of roadside revegetation. Action Alternatives and Recommendations to modify current practices are those considered feasible within the context of present Caltrans operations.

Review Sections are arranged as follows:

Section 2 **Standard Specification 20-2.10**

- A restatement of Standard Specification 20-2.10;
- A synopsis of *University of California Agricultural Experiment Station Bulletin 1842*, "Range-Legume Inoculation and Nitrogen Fixation by Root-Nodule Bacteria";
- A scanned copy of the entire *UC-AES Bulletin 1842*.

Section 3 **Summary Review of Standard Specification 20-2.10**

A synoptic evaluation of Standard Specification 20-2.10 with minimal discussion

Section 4 **Expanded Review of Standard Specification 20-2.10**

A detailed evaluation of Standard Specification 20-2.10 with expanded discussion and reference citations where necessary.

Section 5 **Actions & Recommendations**

Action Alternatives for modifying Standard Specification 20-2.10. Recommendations for modifications to revegetation practices.

Section 6 **References**

A numbered list of all references cited in the document. Throughout the document, references are cited in the format [**1**].

Appendix A **Glossary**

Definitions of technical terms used in the document.

Section 2
Standard Specification 20-2.10

Box 2.1

STANDARD SPECIFICATION

SECTION 20: EROSION CONTROL AND HIGHWAY PLANTING

20-2 MATERIALS

20-2.10 SEED

- Legume seed shall be pellet-inoculated with a viable bacteria compatible for use with that species of seed. All inoculated seed shall be labeled to show the mass of seed, the date of inoculation and the mass and source of inoculant materials.
- Legume seed shall be pellet-inoculated in conformance with the requirements in Bulletin 1842, "Range-Legume Inoculation and Nitrogen Fixation by Root-Nodule Bacteria," of the University of California, Division of Agriculture and Natural Resources. Inoculant shall be added at the rate of 2 kg {2 pounds} of inoculant per 100 kg {100 pounds} of legume seed.
- Inoculated seed shall be sown within 90 days of inoculation.

The present California State Standard Specification 20-2.10 for legume seed inoculation derives from the *University of California Agricultural Experiment Station Bulletin 1842*, "Range-Legume Inoculation and Nitrogen Fixation by Root-Nodule Bacteria," published in 1987 [**118**]. *UC-AES Bulletin 1842* is a revision of the *University of California Agricultural Experiment Station Bulletin 842*, with the same title, published in 1969 [**81**]. **Box 2.1** provides the exact statements made in Standard Specification 20-2.10 regarding legume seed inoculation.

On following pages, the most important portions of *UC-AES Bulletin 1842* are reformatted as:

Box 2.2, Pellet Inoculation Of Legume Seed
Describes necessary materials and procedures for pellet inoculation.

Box 2.3, Specific Relationships Between Host Legumes and Rhizobia
Lists the typical pasture legumes (clover, pea, vetch, alfalfa, and trefoil) associated with different species of *Rhizobium* bacteria typically used for inoculation.

Box 2.4, Seed Inoculation and Field Problems
Lists typical problems associated with inoculant sources, purity, shelf life, storage conditions, site factors, and other considerations.

Following the synoptic boxes, scanned pages of the entire *UC-AES Bulletin 1842* are provided, as well.

Box 2.2

UNIVERSITY OF CALIFORNIA
AGRICULTURAL EXTENSION BULLETIN 1842
PELLET INOCULATION OF LEGUME SEED

Pelleting is an effective method of legume seed inoculation. Each pellet consists of a legume seed, the peat inoculant carrying *Rhizobium* bacteria, an adhesive to stick the inoculant to the seed, and, frequently, a coating of calcium carbonate. Both the adhesive and the calcium carbonate coating increase the survival of the bacteria. The following recommendations are based on experiments in the California Agriculture Experiment Station.

Materials

Inoculant. Root-nodule bacteria for range legumes are most commonly sold in a peat carrier designed to stick to the legume seed. For agronomic legumes such as alfalfa and soybean, other types of granular and liquid inocula can be drilled into the seed bed at the time of planting, but such materials are not currently available for range legumes. The amount of inoculant recommended by each manufacturer will be printed on the package. It usually is most effective to use four times the recommended amount. Never use less than the amount suggested on the package.

Adhesive. Some commercial inoculants are sold with their own separate package of a material that serves as both adhesive and coating. These products are generally very effective, and they are convenient to use. In the absence of such a combination product, one can prepare a very good adhesive from gum arabic. Use a technical grade of gum arabic powder or granules obtainable from laboratory chemical supply companies. Do not purchase gum arabic containing a preservative, because it may harm the root-nodule bacteria.

Coating. When gum arabic is used as an adhesive, the pellet can be coated with calcium carbonate ($CaCO_3$). Suitable products are marketed as lime, calcium carbonate. calcite, enamel whiting, and 280 whiting. Do not use quicklime; it is highly toxic to root-nodule bacteria. The coating should be ground fine so at least 80 percent of it passes through a 200-mesh screen.

Quantities. The amount of pelleting materials needed varies with the size of the seed. For the adhesive, use 2 pounds of inoculant adhesive or 4 pounds of gum arabic powder to 1 gallon of water. This makes about 5 quarts of solution.

Subterranean, Rose, or Crimson Clover: for 100 pounds of seed, use 5 quarts of adhesive solution. Add 50 pounds of calcium carbonate if using gum arabic.

Vetch: for 100 pounds of seed, use 2.5 quarts of adhesive solution. Add 30 pounds of calcium carbonate if using gum Arabic.

Alfalfa, or Bur Clover: for 100 pounds of seed, use 5 quarts of adhesive solution. Add 40 pounds of calcium carbonate if using gum arabic.

Pellet Preparation

1. Use a cement mixer for large quantities of seed. Small lots may be pelleted by hand in a tub or a bucket, or on a smooth floor.

2. Calculate the appropriate quantities of inoculant adhesive or gum arabic and water to use with the quantity of seed to be pelleted.

3. In a separate container, dissolve inoculant adhesive or gum arabic in water. There are two possible methods: either add the powder to the water slowly, while stirring vigorously, or else make a paste by adding half the water to the powder and then dilute with the remaining water. Both substances dissolve overnight in cold water, or in about 30 minutes in hot water (not boiling). Cool the hot solution.

4. Just before pelleting, add the appropriate amount of inoculant and stir to a smooth slurry. This mixture must not stand for more than 30 minutes. Some gum arabic is acidic and will harm the bacteria unless the acid is neutralized by the calcium carbonate as soon as possible. In commercial products that function as both adhesive and coating material, the pH is controlled and is not a problem.

5. Pour the seed into the mixer. Add the gum inoculant mixture and rotate the mixer at high speed, for good tumbling action, until all the seeds are coated.

6. For gum arabic, dump in the calcium carbonate all at once without stopping the mixer and let the mixer run until all the seeds are pelleted.

7. Do not clean the mixer between loads. After the whole job is done, clean the mixer by running a load of water and gravel through it.

8. Pellets are firmer if they age for 24 hours. They will then work better for drill-seeding.

9. Screen the pelleted seed to remove lumps that may clog the seeding equipment. The proportions given above allow for an excess of calcium carbonate, because the stickiness of the adhesive may vary slightly in different lots.

10. To prevent injury to the pellet coating, remove vigorous agitators from seeding equipment.

Box 2.3

UNIVERSITY OF CALIFORNIA
AGRICULTURAL EXTENSION BULLETIN 1842
SPECIFIC RELATIONSHIPS BETWEEN HOST LEGUMES AND RHIZOBIA

The relationship of a species or strain of *Rhizobium* to certain legumes often is very specific. The classification of *Rhizobium* into cross-inoculation groups is somewhat arbitrary, since sometimes bacteria can infect legumes outside the accepted host groups, and not every strain of a given species of *Rhizobium* invades all of the legumes in the group.

1. CLOVER GROUP, nodulated by *Rhizobium leguminosarum* biov. *trifolii*

Alsike Clover	*Trifolium hybridum*
Berseem Clover	*Trifolium alexandrinum*
Crimson Clover	*Trifolium incarnatum*
Ladino Clover	*Trifolium repens giganteum*
Red Clover	*Trifolium pratense*
Rose Clover	*Trifolium hirtum*
Strawberry Clover	*Trifolium fragiferum*
Subterranean Clover	*Trifolium subterraneum*
White Clover	*Trifolium repens*

2. PEA AND VETCH GROUP, nodulated by *Rhizobium leguminosarum* biov. *viceae*

Field Pea and Garden Pea	*Pisum sativum*
Common Vetch	*Vicia sativa*
Hairy Vetch	*Vicia villosa*
Horsebean	*Vicia faba*
Purple Vetch	*Vicia benghalensis*
Woollypod Vetch	*Vicia dasycarpa*

3. ALFALFA GROUP, nodulated by *Rhizobium meliloti*

Alfalfa	*Medicago sativa*
Barrel Medic	*Medicago tribuloides*
Black Medic	*Medicago lupulina*
Bur Clover	*Medicago hispida*
Fenugreek	*Trigonella foenum-graecum*
Hubam Clover	*Melilotus alba annua*
Sourclover	*Melilotus indica*
White Sweetclover	*Melilotus alba*
Yellow Sweetclover	*Melilotus officinalis*

There are a number of legume species outside the seven recognized groups. Each of these legumes depends on its own particular strain of *Rhizobium* for effective nodulation. The following species among these ungrouped legumes are important in California:

Big Trefoil	*Lotus uliginosus*
Birdsfoot Trefoil prostrate	*Lotus tenuis*
Birdsfoot Trefoil upright	*Lotus corniculatus*

Box 2.4

UNIVERSITY OF CALIFORNIA AGRICULTURAL EXTENSION BULLETIN 1842

SEED INOCULATION AND FIELD PROBLEMS

1. Inoculated legume seeds can be obtained by one of three methods:

(A) by purchasing pre-inoculated seeds
(B) by contracting for custom inoculation services
(C) by the rancher pelleting seeds on-site.

Method A is not recommended because the shelf-life of rhizobia on inoculated seeds is very short.

Method B can be successful, but the supplier and the expenses must be investigated carefully.

This bulletin is directed toward helping individuals use Method C to pellet their own legume seeds.

2. Select a good commercial peat inoculant that contains root-nodule bacteria specific for the legume to be planted. Make sure the legume species is named on the label. No other inoculant is suitable.

3. Check the freshness of the inoculant by referring to the expiration date printed on the container. The inoculant is a living culture of root nodule bacteria that can be killed by drying and by high temperatures. Make sure the culture has been stored under refrigeration. Poor storage conditions can cause nodulation failure by reducing the number of viable bacteria in the inoculant.

4. Use the inoculant at the rate the manufacturer recommends on the package, or, even better, at four times the recommended rate. Never use a lower amount, or there will be too few bacteria on each seed to produce good nodulation.

5. Mix the inoculant thoroughly with the seed. Closely follow the directions on the package or those under Pellet Inoculation of Legume Seed in this bulletin.

6. Hold the inoculated seed in a cool, shady place, and plant as soon as possible into a seedbed that will receive a germinating rain in the very near future. If possible, plant into a moist seedbed after the first fall rain. Never plant in the summer months. Always remember that drying kills bacteria in the inoculant and reduces nodulation.

7. Do not mix acid fertilizers with inoculated seeds, and do not sow seeds in contact with such fertilizers. The acidity may kill most or all of the root-nodule bacteria.

8. For the same reason, do not mix the seeds with fertilizers that contain trace elements, unless the manufacturer has specifically formulated and recommended the fertilizer product for that use.

9. Do not use herbicides, fungicides, or any other pesticides when planting inoculated seeds. Many of these poisons are highly toxic to root-nodule bacteria.

10. Check the acidity of the soil where inoculated seeds will be planted. Legumes usually fail to nodulate when the soil is more acid (lower pH) than pH 5.2. If the soil pH is too low, an appropriate amount of lime should be added to the soil at the time of planting. Drilling lime in with the seed is suitable, but coating seeds with calcium carbonate accomplishes the same thing and is compatible with broadcasting the seeds.

11. Make sure the soil contains adequate amounts of plant nutrients. A legume suffering from a deficiency of any nutrient other than nitrogen cannot benefit fully from inoculation. To maximize grazing potentials the legumes must have an adequate supply of available phosphorus and sulfur, the elements most commonly deficient on California rangelands. Many pasture legumes such as subterranean clover are less competitive than grasses for soil nutrients.

12. Do not use nitrogen fertilizers when planting legumes in a pasture. Well-nodulated legumes do not need any nitrogen from the soil, and nitrogenous fertilizers usually give grasses a competitive advantage over legumes. Moreover, ammonia, nitrates, and nitrites inhibit legume nodulation under most circumstances.

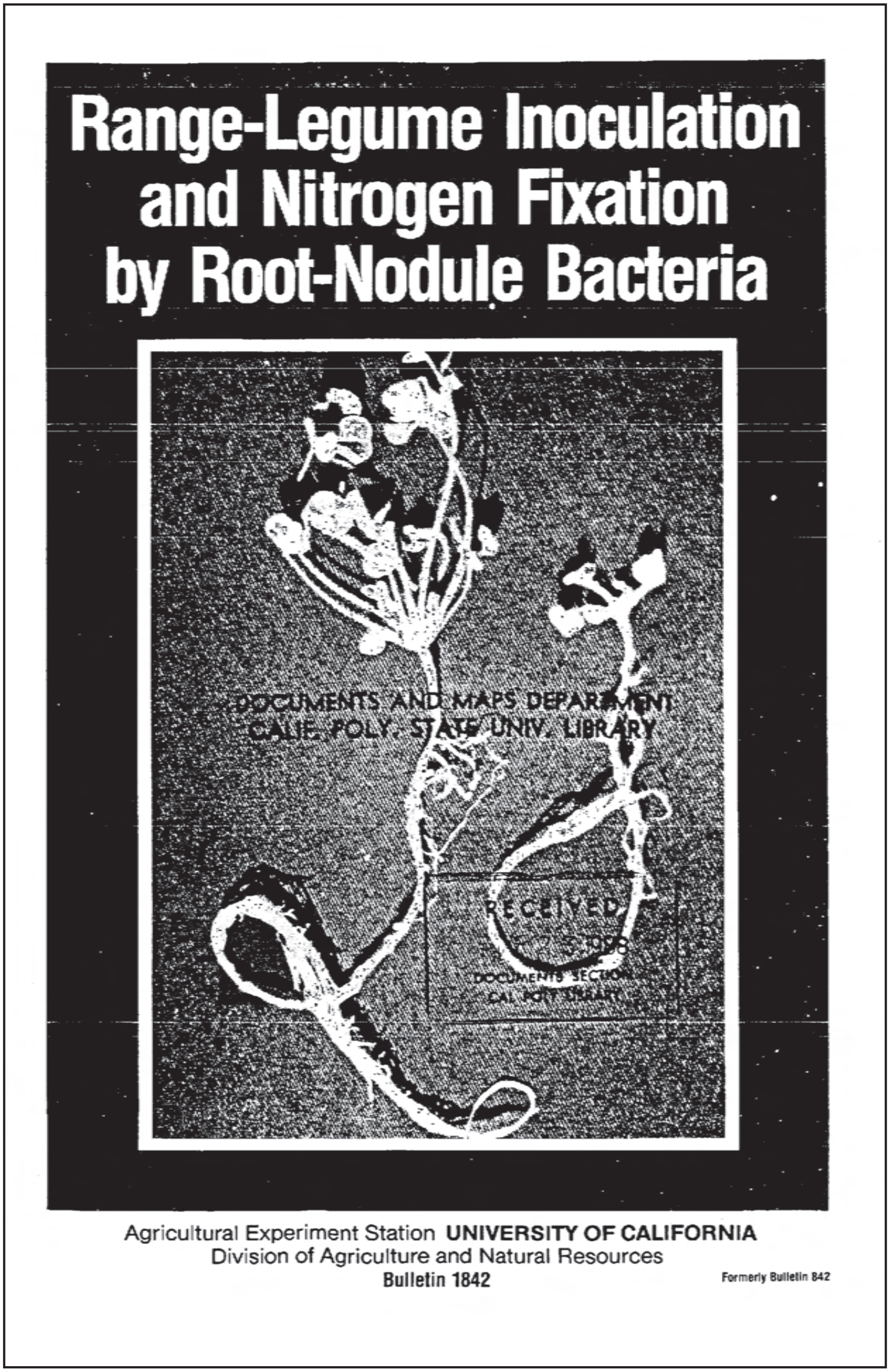
13. Maintain adequate pasture management. In a grass-dominated pasture direct every effort toward making the environment more favorable for legumes than for grasses. A good legume stand should have at least 20 plants per square foot. Grazing the pasture favors legumes by reducing competition from grasses. Reduce grazing pressure only while the legumes are flowering and setting seed. Graze the pasture quite heavily in summer after the legumes are dead.

14. Dig a few seedlings after the legumes have produced three or four leaves. The type and pattern of nodulation can give useful information:

- A few large nodules on the crown or upper root indicate early, effective nodulation, provided the nodules are pink inside.
- Lack of nodulation usually indicates some fault in the inoculation or sowing technique. Review the above instructions.
- Many small, white nodules scattered over the entire root system also suggest an inoculation problem, and indicate the presence of ineffective root-nodule bacteria in the soil. A good peat inoculant and proper inoculation technique should eliminate this problem.

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842*.

Cover



Range-Legume Inoculation and Nitrogen Fixation by Root-Nodule Bacteria

Agricultural Experiment Station **UNIVERSITY OF CALIFORNIA**
Division of Agriculture and Natural Resources
Bulletin 1842

Formerly Bulletin 842

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842.*

Cover (inside)

THE AUTHORS

D. A. Phillips and W. A. Williams are both Agronomists,
Agricultural Experiment Station,
and Professors, Department of Agronomy and Range Science,
University of California, Davis.

PREFACE

An important characteristic of agricultural legumes is that they can use elemental atmospheric nitrogen to make proteins. This process, "nitrogen fixation," allows legumes to use the large reservoir of nitrogen in air—nearly 80 percent of the atmosphere—and add nitrogen compounds to the soil for other plants. This means that legumes do not need nitrogen fertilizer and thus can cost the grower less than other crops. Legumes cannot, however, fix nitrogen by themselves. An effective strain of root-nodule bacteria must be present. These bacteria, known as rhizobia, form root nodules on the legume, and atmospheric nitrogen is fixed within these nodules for use by the plant.

The successful establishment of legumes, particularly in a pasture mix for grazing, depends on effective nodulation. This can be obtained by inoculating the seed with an appropriate strain of root-nodule bacteria. Ways to inoculate seed and measures that help to avoid inoculation failure are given here in boxed items: *Seed Inoculation and Field Problems* and *Pellet Inoculation of Legume Seed*. All of the recommendations given here are of critical importance. There is no economical way to inoculate a field after planting.

Faulty inoculation usually results in full or partial failure of the legume stand. From this cause alone, California growers waste many thousands of dollars' worth of range-legume seed, labor, and fertilizer every year.

Cover photo: Woogenellup subterranean clover plants 42 days old. Vigorous plant at left was grown from *Rhizobium*-inoculated seed; small plant at right from uninoculated seed. Both were planted at the same time and only a few feet apart in Yolo County.

2.5m-rev-3/87-WJC/FB

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842.*

Page 1

Range-Legume Inoculation and Nitrogen Fixation by Root-Nodule Bacteria

Introduction

Nitrogen is an essential constituent of amino acids and proteins in animals and plants. Most plants contain 1 to 4 percent nitrogen on a dry weight basis, so large quantities of this element are required for plant growth. Grasses must obtain all their nitrogen from the soil or from fertilizer supplied by the grower. Legumes, however, such as various clovers and vetch, can use elemental atmospheric nitrogen to make proteins if appropriate root-nodule bacteria are present. In a grass-clover mixture, the grass plants use the soil nitrogen while the clover obtains nitrogen primarily from the air (78 percent of the atmosphere is elemental nitrogen). When other soil nutrients, such as phosphorus and sulfur, do not limit plant growth, total forage production can often be increased significantly through the use of an appropriate strain of nitrogen-fixing root-nodule bacteria. This Bulletin provides the information required to inoculate legumes successfully with root-nodule bacteria and to use them productively in a dryland pasture environment.

Traditionally, nitrogen-fixing root-nodule bacteria are classified in the genus *Rhizobium*, and as a group they are called rhizobia. Actually the rhizobia, and not the legume, are responsible for fixing elemental nitrogen into ammonia. The bacteria are nourished within the root nodule by plant carbohydrates, and the ammonia excreted by the rhizobia is converted into amino acids by the plant before it can reach the soil environment where other organisms could use it. This functional association, a symbiosis, has evolved over many, many years, and each root nodule represents the final product of a complex interaction between the legume and the rhizobia.

Burns and Hardy (1975) estimated that on a global scale, biological nitrogen fixation, primarily from the *Rhizobium*-legume association, provides plants with 175 million tons of nitrogen annually. That figure compares favorably to the 30 million tons of nitrogen fertilizer produced annually by industry. The nitrogen derived from rhizobia by fixation is a nearly cost-free input that can increase the productivity of a pasture system.

Under California rangeland conditions, effectively nodulated legumes can fix significant quantities of nitrogen, but of course the amounts are not as great as those reported in legumes such as alfalfa cultivated under optimum conditions. Phillips and Bennett (1978) measured nitrogen fixation rates as high as 180 pounds per acre in pure stands of subterranean clover, but more typical values for several range legumes under various conditions are shown in table 1. A

Table 1. Estimates of nitrogen fixation under California rangeland conditions

Plant	County	Soil	Nitrogen	Reference
			lb/a	
Rose clover	Stanislaus	Snelling sandy loam	50	Williams, Love, and Berry 1957
Rose clover	Placer	Placentia sandy loam	60	Martin, Williams, and Johnson 1957
Subterranean clover	Humboldt	Wilder gravelly loam	45	Williams, Lenz, and Murphy 1954
Subterranean clover	Mendocino	Laughlin loam	61	Phillips, Jones, and Center 1987
Subterranean clover	Mendocino	Yorkville clay	56	Phillips, Jones, and Center 1987
Subterranean clover	Mendocino	Sutherlin loam	54	Phillips, Jones, and Center 1987

1

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842.*

Page 2

reasonable average for a good stand of range legumes might be at least 50 pounds per acre during one growing season, the equivalent of 250 pounds per acre of ammonium sulfate. The cost of the root-nodule inoculation is about $10 per acre, but inoculation is critical to a successful stand of legumes in California annual grasslands (Jones, Burton, and Vaughn 1978).

Root Nodules and Nitrogen Fixation

Recent advances in our understanding of the genetics, biochemistry, and physiology of interactions between legumes and rhizobia have contributed to an exponentially growing body of information on how root nodules develop and function. One can realistically predict that such fundamental information will lead to increases in symbiotic nitrogen fixation at the agronomic and rangeland level. The following discussion is based on information reported by many individ-

Seed Inoculation

1. Inoculated legume seeds can be obtained by one of three methods: (A) by purchasing pre-inoculated seeds, (B) by contracting for custom inoculation services, or (C) by the rancher pelleting seeds on-site. Method A is not recommended because the shelflife of rhizobia on inoculated seeds is very short. Method B can be successful, but the supplier and the expenses must be investigated carefully. This bulletin is directed toward helping individuals use Method C to pellet their own legume seeds.

2. Select a good commercial peat inoculant that contains root-nodule bacteria specific for the legume to be planted. Make sure the legume species is named on the label. No other inoculant is suitable.

3. Check the freshness of the inoculant by referring to the expiration date printed on the container. The inoculant is a living culture of root-nodule bacteria that can be killed by drying and by high temperatures. Make sure the culture has been stored under refrigeration. Poor storage conditions can cause nodulation failure by reducing the number of viable bacteria in the inoculant.

4. Use the inoculant at the rate the manufacturer recommends on the package, or, even better, at four times the recommended rate. Never use a lower amount, or there will be too few bacteria on each seed to produce good nodulation.

5. Mix the inoculant thoroughly with the seed. Closely follow the directions on the package or those under *Pellet Inoculation of Legume Seed* in this bulletin.

6. Hold the inoculated seed in a cool, shady place, and plant as soon as possible into a seedbed that will receive a germinating rain in the very near future. If possible, plant into a moist seedbed after the first fall rain. Never plant in the summer months. Always remember that drying kills bacteria in the inoculant and reduces nodulation.

7. Do not mix acid fertilizers with inoculated seeds, and do not sow seeds in contact with such fertilizers. The acidity may kill most or all of the root-nodule bacteria.

8. For the same reason, do not mix the seeds with fertilizers that contain trace elements, unless the manufacturer has specifically formulated and recommended the fertilizer product for that use.

9. Do not use herbicides, fungicides, or any other pesticides when planting inoculated seeds.

2

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842.*

Page 3

ual scientists at a recent international conference on nitrogen fixation (Evans, Bottomley, and Newton 1985).

Root nodule development

For a root nodule to form on a legume, a complex interaction among bacterial and plant genes must take place. Some estimates suggest that 10 to 25 bacterial genes may be involved in the process, but little information is available on the number of plant genes involved. Whether or not a particular strain of rhizobia will form a nodule on the root of a given legume depends on the type of host-specificity-of-nodulation (*hsn*) genes present. The *hsn* genes are carried by the bacteria on large segments of extrachromosomal DNA known as plasmids, which can be transferred sexually between different rhizobia. Under laboratory conditions, it is possible to alter the capacity of a *Rhizobium* strain to infect various legumes, but whether such changes occur under field conditions is unknown. The *hsn* genes are involved in such early steps of bacterial infection as attachment, root hair curling, and root hair infection. A closely related group of nodulation (*nod*) genes in the bacteria is also active during the early stages of infection and root nodule

and Field Problems

Many of these poisons are highly toxic to root-nodule bacteria.

10. Check the acidity of the soil where inoculated seeds will be planted. Legumes usually fail to nodulate when the soil is more acid (lower pH) than pH 5.2. If the soil pH is too low, an appropriate amount of lime should be added to the soil at the time of planting. Drilling lime in with the seed is suitable, but coating seeds with calcium carbonate accomplishes the same thing, and is compatible with broadcasting the seeds.

11. Make sure the soil contains adequate amounts of plant nutrients. A legume suffering from a deficiency of any nutrient other than nitrogen cannot benefit fully from inoculation. To maximize grazing potentials the legumes must have an adequate supply of available phosphorus and sulfur, the elements most commonly deficient on California rangelands. Many pasture legumes such as subterranean clover are less competitive than grasses for soil nutrients.

12. Do not use nitrogen fertilizers when planting legumes in a pasture. Well-nodulated legumes do not need any nitrogen from the soil, and nitrogenous fertilizers usually give grasses a competitive advantage over legumes. Moreover, ammonia, nitrates, and nitrites inhibit legume nodulation under most circumstances.

13. Maintain adequate pasture management. In a grass-dominated pasture, direct every effort toward making the environment more favorable for legumes than for grasses. A good legume stand should have at least 20 plants per square foot. Grazing the pasture favors legumes by reducing competition from grasses. Reduce grazing pressure only while the legumes are flowering and setting seed. Graze the pasture quite heavily in summer after the legumes are dead.

14. Dig a few seedlings after the legumes have produced three or four leaves. The type and pattern of nodulation can give useful information:

- A few large nodules on the crown or upper root indicate early, effective nodulation, provided the nodules are pink inside.
- Lack of nodulation usually indicates some fault in the inoculation or sowing technique. Review the above instructions.
- Many small, white nodules scattered over the entire root system also suggest an inoculation problem, and indicate the presence of ineffective root-nodule bacteria in the soil. A good peat inoculant and proper inoculation technique should eliminate this problem.

3

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842.*

Page 4

growth. Evidence now indicates that several of the *nod* genes are activated by a specific chemical product from the host plant. Different chemicals may be generated by different legumes, or there may be some common signal. By understanding this interaction, agronomists may be able to use chemicals that will prevent poor rhizobia present in the soil from forming root nodules while allowing superior bacteria pelleted onto the seed to form root nodules that will have higher rates of nitrogen fixation.

As rhizobia enter into a root hair, they form an infection thread that penetrates into the body of the root where a tumor-like proliferation of root cells results. Within the young nodule, bacteria are released from the infection thread into the cytoplasm of root cells. The plant surrounds these invasive bacteria with a membrane, and the rhizobia differentiate into strangely shaped cells called bacteroids that are capable of fixing atmospheric nitrogen.

Root nodule function

Although the biochemical and physiological functions of root nodules are reasonably well understood, there is little information available on genetic control of the process. Most experts believe that many more genes are involved in the functioning of root nodules than in their formation. When the genes affecting root nodule functioning are understood, it should be possible to

Pellet Inoculation

Pelleting is an effective method of legume seed inoculation. Each pellet consists of a legume seed, the peat inoculant carrying *Rhizobium* bacteria, an adhesive to stick the inoculant to the seed, and, frequently, a coating of calcium carbonate. Both the adhesive and the calcium carbonate coating increase the survival of the bacteria. The following recommendations are based on experiments in the California Agricultural Experiment Station.

Materials

Inoculant. Root-nodule bacteria for range legumes are most commonly sold in a peat carrier designed to stick to the legume seed. For agronomic legumes such as alfalfa and soybean, other types of granular and liquid inocula can be drilled into the seed bed at the time of planting, but such materials are not currently available for range legumes. The amount of inoculant recommended by each manufacturer will be printed on the package. It usually is most effective to use four times the recommended amount. Never use less than the amount suggested on the package.

Adhesive. Some commercial inoculants are sold with their own separate package of a material that serves as both adhesive and coating. These products are generally very effective, and they are convenient to use.

In the absence of such a combination product, one can prepare a very good adhesive from gum arabic. Use a technical grade of gum arabic powder or granules obtainable from laboratory chemical supply companies. Do not purchase gum arabic containing a preservative, because it may harm the root-nodule bacteria.

Coating. When gum arabic is used as an adhesive, the pellet can be coated with calcium carbonate ($CaCO_3$). Suitable products are marketed as lime, calcium carbonate, calcite, enamel whiting, and 280 whiting. Do not use quicklime; it is highly toxic to root-nodule bacteria. The coating should be ground fine so at least 80 percent of it passes through a 200-mesh screen.

Quantities. The amount of pelleting materials needed varies with the size of the seed. For the adhesive, use 2 pounds of inoculant adhesive or 4 pounds of gum arabic powder to 1 gallon of water. This makes about 5 quarts of solution.

- Subterranean, rose, or crimson clover: for 100 pounds of seed, use 5 quarts of adhesive solution. Add 50 pounds of calcium carbonate if using gum arabic.
- Vetch: for 100 pounds of seed, use 2½ quarts of adhesive solution. Add 30 pounds

4

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842*.

Page 5

improve nitrogen fixation in agronomic and rangeland environments by modifying specific physiological processes. It already has been demonstrated that a mutation in soybean rhizobia can increase seed yield by 12 percent under California field conditions (Williams and Phillips 1983), and, presumably, similarly improved rhizobia will be developed for range legumes. Genetic modification of the host plant has successfully increased nitrogen fixation in alfalfa and improved forage yield and quality (Phillips, Demment, and Teuber 1985). The techniques used on alfalfa should be easy to adapt to range legumes, but an equivalent 10-year program of improvement would be required.

Critical aspects of root-nodule functioning include:

1. Atmospheric nitrogen gas diffuses into the *Rhizobium* bacteroids where it is converted to ammonia and excreted into the plant cell cytoplasm. The ammonia is incorporated into organic compounds by the plant and is transported to other parts of the plant without being released into the surrounding soil. The nitrogen from the symbiotic fixation process initially is available only to the legume and not to the grass component in a rangeland environment.

2. Oxygen gas, which is present in the atmosphere, inhibits the nitrogenase enzyme complex

of Legume Seed

of calcium carbonate if using gum arabic.

- Alfalfa or bur clover: for 100 pounds of seed, use 5 quarts of adhesive solution. Add 40 pounds of calcium carbonate if using gum arabic.

Preparing the pellets

1. Use a cement mixer for large quantities of seed. Small lots may be pelleted by hand in a tub or a bucket, or on a smooth floor.

2. Calculate the appropriate quantities of inoculant adhesive or gum arabic and water to use with the quantity of seed to be pelleted.

3. In a separate container, dissolve inoculant adhesive or gum arabic in water. There are two possible methods: either add the powder to the water slowly, while stirring vigorously, or else make a paste by adding half the water to the powder and then dilute with the remaining water. Both substances dissolve overnight in cold water, or in about 30 minutes in hot water (not boiling). Cool the hot solution.

4. Just before pelleting, add the appropriate amount of inoculant and stir to a smooth slurry. This mixture must not stand for more than 30 minutes. Some gum arabic is acidic and will harm the bacteria unless the acid is neutralized by the calcium carbonate as soon as possible. In commercial products that function as both adhesive and coating material, the pH is controlled and is not a problem.

5. Pour the seed into the mixer. Add the gum inoculant mixture and rotate the mixer at high speed, for good tumbling action, until all the seeds are coated.

6. For gum arabic, dump in the calcium carbonate all at once without stopping the mixer and let the mixer run until all the seeds are pelleted.

7. Do not clean the mixer between loads. After the whole job is done, clean the mixer by running a load of water and gravel through it.

8. Pellets are firmer if they age for 24 hours. They will then work better for drill-seeding.

9. Screen the pelleted seed to remove lumps that may clog the seeding equipment. The proportions given above allow for an excess of calcium carbonate, because the stickiness of the adhesive may vary slightly in different lots.

10. To prevent injury to the pellet coating, remove vigorous agitators from seeding equipment.

5

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842.*

Page 6

responsible for reducing elemental nitrogen to ammonia. However, oxygen is required by rhizobia to produce the energy needed to operate the nitrogenase system. To overcome this problem, plant cells in root nodules produce a protein, leghemoglobin, which transports oxygen into rhizobia while maintaining a low concentration of the free oxygen that could otherwise inhibit nitrogenase. This system is especially important in soils where changing water content can greatly alter the availability of oxygen around the root nodule. The leghemoglobin in healthy root nodules is bright red and gives the nodules a pink outer appearance.

3. *Rhizobium* bacteroids in root nodules depend on the host legume for a supply of carbon compounds that can be used with oxygen to provide energy for nitrogen fixation. The carbon compounds come from photosynthesis in the plant shoot, and less carbon is available during periods of low temperature or cloudy weather, when plants are photosynthesizing less and growing more slowly. Thus nitrogen fixation is closely coupled to growth of the host legume, and there is no chance for the legume to fix extra nitrogen for adjacent grass plants in a rangeland environment.

Root nodule senescence

After many weeks or months of nitrogen-fixing activity, roots and root nodules senesce and break down. The nitrogen previously fixed then becomes available to other plants. Rhizobia that remain in the infection thread, having not been converted into nitrogen-fixing bacteroids, multiply rapidly in the senescing nodule tissue before being released into the soil. Neither the normal rod forms of rhizobia nor the bacteroids produce spores or other resting bodies that might protect the bacteria from unfavorable conditions in the soil. Therefore, it is believed that rhizobia are always subject to death and possibly to mutational pressures from drying. The hot, dry environment of rangeland soils during the California summer would seem to present an extraordinarily inhospitable situation for rhizobia, but the organisms persist from early summer until late fall when annual legumes germinate again. Perhaps the bacteria are modified by some type of osmotic adjustment during the senescence process. Understanding such a phenomenon could provide another agronomic tool, in addition to the inoculation procedure described here, for controlling the particular *Rhizobium* strain present in a given soil environment.

Specific Relationships between Host Legumes and Rhizobia

The relationship of a species or strain of *Rhizobium* to certain legumes often is very specific.

Infection

Usually, one species of *Rhizobium* can form nodules on many of the legumes of one or more genera. Thus, the agriculturally important legumes have been classified into seven so-called cross-inoculation groups: clover, alfalfa, pea and vetch, bean, lupine, soybean, and cowpea. Each group of legumes is commonly compatible with several related strains of one *Rhizobium* species. Moreover, rhizobia of this one species usually do not invade legumes outside their particular group.

The following list gives only the more important forage legumes in three of the cross-inoculation groups:

1. The clover group, nodulated by *Rhizobium leguminosarum* biov. *trifolii*
Alsike clover, *Trifolium hybridum*
Berseem clover, *T. alexandrinum*
Crimson clover, *T. incarnatum*
Ladino clover, *T. repens giganteum*
Red clover, *T. pratense*
Rose clover, *T. hirtum*
Strawberry clover, *T. fragiferum*
Subterranean clover, *T. subterraneum*
White clover, *T. repens*

2. The alfalfa group, nodulated by *Rhizobium meliloti*
Alfalfa, *Medicago sativa*
Barrel medic, *M. tribuloides*
Black medic, *M. lupulina*
Bur clover, *M. hispida*
Fenugreek, *Trigonella foenumgraecum*
Hubam clover, *Melilotus albu annua*

6

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842.*

Page 7

Sourclover, *M. indica*
White sweetclover, *M. alba*
Yellow sweetclover, *M. officinalis*

3. The pea and vetch group, nodulated by *Rhizobium leguminosarum* biov. *viceae*
Field pea and garden pea, *Pisum sativum*
Common vetch, *Vicia sativa*
Hairy vetch, *V. villosa*
Horsebean, *V. faba*
Purple vetch, *V. benghalensis*
Woollypod vetch, *V. dasycarpa*

The classification of *Rhizobium* into cross-inoculation groups is somewhat arbitrary, since sometimes bacteria can infect legumes outside the accepted host groups, and not every strain of the given species of *Rhizobium* invades all of the legumes in the group.

There are a number of legume species outside the seven recognized groups. Each of these legumes depends on its own particular strain of *Rhizobium* for effective nodulation. The following species among these ungrouped legumes are important in California:

Big trefoil, *Lotus uliginosus*
Birdsfoot trefoil, prostrate, *L. tenuis*
Birdsfoot trefoil, upright, *L. corniculatus*

Effectiveness of rhizobial strains

As a further complication, each *Rhizobium* species contains strains that differ in ability to fix nitrogen in symbiosis with a given species or variety of legume. Also, a strain of rhizobia that fixes little nitrogen with a certain legume may fix a large amount with another legume in the same cross-inoculation group. As an example, some strains of *Rhizobium* that nodulate both red clover and subterranean clover fix much more nitrogen with the red clover than with the subterranean clover. The reverse is true with some other strains. Such variability in the effectiveness of rhizobial strains emphasizes the necessity for using a specific inoculant for each legume.

Selection of rhizobial strains

Strains of rhizobia to be used as inoculants can be isolated from nodules on vigorous legumes in a field. Preferably, these strains should be selected from the general geographic region in which the inoculants are to be used. Strains so selected then can be compared with each other on seedlings grown aseptically under controlled conditions (table 2). The best strains are tested further in the field.

Table 2. Laboratory tests of selected strains of *Rhizobium leguminosarum* biov. *trifolii**

Rhizobium strain and origin	Foliage nitrogen content	Foliage weight green†
	percentage	*mg/plant*
Host plant: Subterranean clover		
None. Check plants with nitrogen	3.05	239a
RH 32, Sonoma County	3.09	213a
RH 24, Hopland Field Station	2.55	153b
RH 45, Humboldt County	2.16	124b
RH 48, Humboldt County	1.52	70c
None. Check plants without nitrogen	0.94	54c
Host plant: Rose clover		
None. Check plants with nitrogen	2.67	317a
RH 36, Yolo County	2.83	134b
RH 39, Yolo County	1.93	115c
RH 28, Sonoma County	1.46	64d
RH 24, Hopland Field Station	1.27	57d
None. Check plants without nitrogen	1.06	43d

* Seedlings grown 42 days at 75° F in test tubes with nutrient solutions. Data in this table were reported in the original Bulletin 842 by Holland, Street, and Williams (1969).
† Average of six replicate plants. Values followed by different letters (a,b,c,d) are significantly different at $P < 0.05$.

7

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842.*

Page 8

Resident rhizobia in a soil

Root-nodule bacteria occur in the soil wherever legumes normally grow, but these resident rhizobia may not be effective nitrogen-fixers for a newly planted legume. An abundant soil population of invasive resident rhizobia will nodulate young legume roots as soon as they start to grow. If the legume seed carries only a few rhizobia of the desirable strain, these few cannot compete with the large numbers of resident soil rhizobia, and ineffectively nodulated plants will result. Competition by resident rhizobia is common on California rangelands because of the prevalence of native legumes and their associated rhizobia.

Table 3 shows how competition between resident rhizobia and those inoculant rhizobia affected a planting of subterranean clover. Rhizobia were abundant in the soil and produced many small, ineffective nodules scattered over the root system of the clover, both when the seed was not inoculated and when it was inoculated at the rate of 900 rhizobia per seed. However, a much heavier application of the same inoculant resulted in large and effective nodules on the upper parts of the root, near the seed. In this case, 12 effective nodules per plant fixed a far greater amount of nitrogen than did 43 to 56 ineffective nodules. Total forage protein in the heavily inoculated plot was 12 times that produced in the plot with light inoculum, and 16 times that produced in the plot without inoculum. A graphic comparison between inoculated and uninoculated clover plants is shown in figure 1.

Obviously the nodulation of a legume crop should not be left to chance, in view of the many possibilities of failure. It pays to inoculate seed, it is essential that one use the correct inoculant for the particular legume being planted, and it is equally essential that one use enough inoculant to ensure early and effective nodulation.

Table 3. Effects of inoculation on subterranean clover*

Inoculant bacteria per seed	Nodulation of 50 plants from each treatment†			Yield‡	
	Nodules on crown	Nodules elsewhere	Nodules on root system	Nitrogen content§	Foliage dry weight
	—no. of plants—		*per plant*	*percentage*	*g/plot¶*
0	0	50	56	1.9	3.3
900	0	50	43	2.0	4.2
21,000	43	7	12	3.7	27.3

* Before inoculation all the seeds were surface-sterilized with ethanol and hydrogen peroxide, drained, washed three times in sterile water, and dried under aseptic conditions. Plants were grown 120 days from seed in field plots 3 feet square, separated by buffer areas in the Sutherlin soil series, Hopland Field Station, Mendocino County. Data in this table were reported in the original Bulletin 842 by Holland, Street, and Williams (1969).

† Ten plants selected at random from each of five replicate plots.

‡ There were three 3-foot rows in a plot. Each treatment was replicated in five plots in a randomized block. The center row of each plot was harvested for the yield data.

§ Analysis of trifoliate leaves only.

¶ Difference for testing significance at the 1 percent level = 19.9 grams.

8

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842*.

Page 9

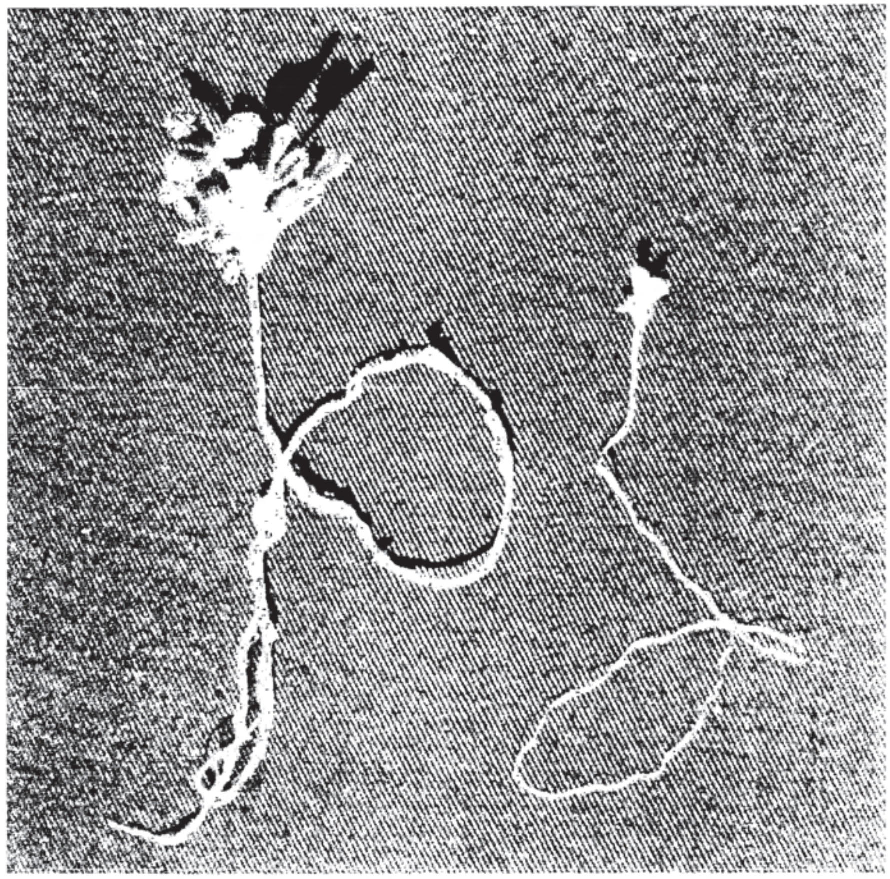


Figure 1. Sirint rose clover 42 days after planting. *Left, Rhizobium* inoculant used; plant grew vigorously. *Right,* uninoculated plants lacked effective nodules and died of nitrogen deficiency.

Practical Considerations

Inoculation methods

Root-nodule bacteria must be alive when the seed is planted and when it germinates. Commercial inoculum that provides highly effective rhizobia is most desirable. Ineffective rhizobia can lead to death of clover plants (fig. 2). Survival of the rhizobia is the most important consideration at every step of handling and storing the inoculant. Failure to consider this may result in poor nodulation and possibly in the death of the legume. Death of rhizobia on the seed before germination probably is responsible for 99 percent of inoculation failures.

Several methods have been developed to maximize survival of rhizobia on legume seeds, but not all techniques are suitable for California rangelands. Older slurry methods of inoculation often produced seeds with only a few rhizobia, and those were killed quickly by desiccation. Australian agricultural scientists developed pelleting methods that used calcium carbonate to

9

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842.*

Page 10

protect the rhizobia from acid soil conditions (Loneragan et al. 1955), and gum arabic, an exudate from the bark of acacia trees, to stick peat inoculum to seeds and to protect rhizobia from desiccation (Brockwell 1962). A pelleting method of inoculation similar to that given under *Pellet Inoculation of Legume Seed* is still the preferred method in Australia for pasture or range legumes where seeding rates are relatively low (Brockwell et al. 1980). However, both in Australia and in the U.S., solid and liquid inoculants are suitable for legumes that must be planted at high seeding rates and can be sown in moist seed beds.

No one can predict the exact number of rhizobia each seed will require to produce effective nodulation. Some experts say that 1,000 rhizobia per seed is an adequate number, but any estimate depends on the environment of the seed bed. The longer a seed remains in a hot, dry seed bed before germinating, the more rhizobia are needed for effective nodulation. Some rhizobia will die whenever they have been inoculated onto a legume seed. The safest goal for a rancher is to place as many rhizobia as possible on each seed.

Identifying an ideal inoculation method for every situation is impossible. The pelleting technique outlined under *Pellet Inoculation of Legume Seed* should produce satisfactory results for all persons who follow the directions carefully and purchase a high-quality peat inoculant. Occasionally, various companies offer commercial inoculation services, but ranchers should consider such opportunities carefully. Frequently, custom inoculation services that use seeds provided by a rancher produce pelleted seeds with

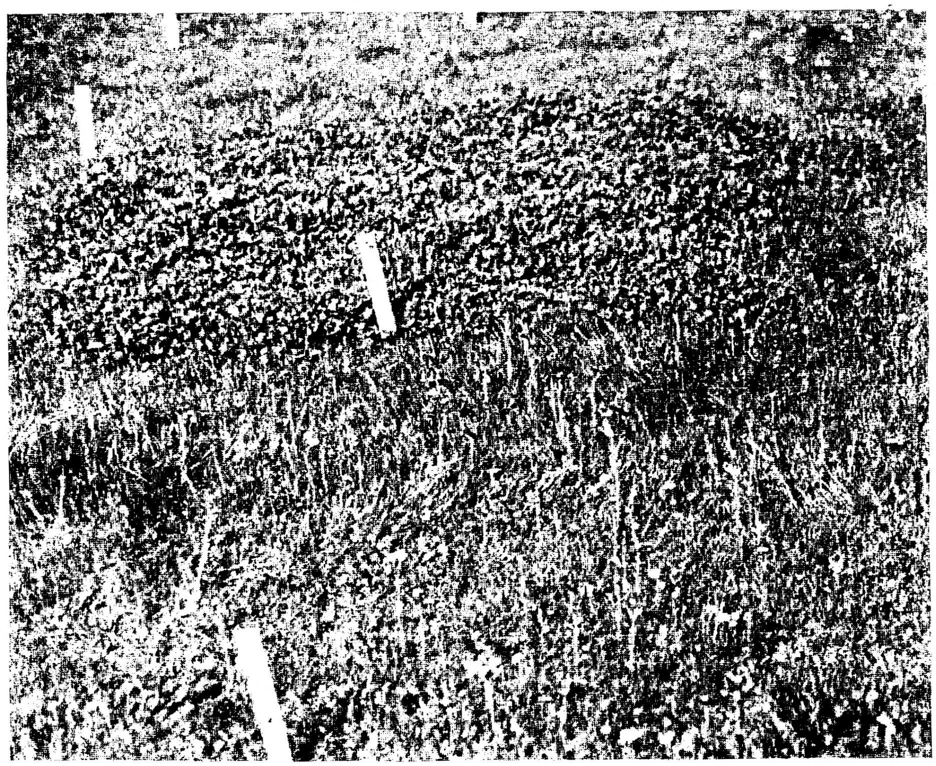


Figure 2. The effect of *Rhizobium* on clover growth. All plots received phosphorus plus sulfur fertilizer and subclover seeds in October. The photograph was taken the following March. Only the plot with extensive clover growth received an effective *Rhizobium* strain in the pellet inoculum. Other plots received an ineffective strain or no *Rhizobium*.

10

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842.*

Page 11

satisfactory to outstanding numbers of bacteria on each seed (table 4). However, tests with the preinoculated seeds often sold by commercial seed companies have shown that the rhizobia may be dead or absent at the time of sale, both in Australia (Brockwell et al. 1975) and in California (B. L. Kay, J. E. Street, M. B. Jones, and D. A. Phillips, unpublished data). The difference apparently is related to delays between preinoculation and sale.

Ideally, we would identify suitable peat inoculants and companies that have satisfactory custom inoculation records. Unfortunately, the products and the services change so rapidly any such information could soon be out of date. An individual rancher who wants the latest information on these products and services should contact a local University of California Cooperative Extension Farm Advisor.

Soil factors critical for rhizobia

As living organisms, rhizobia cannot function in severe soil environments. Rhizobia function best in the pH 6.4 to 7.2 range. At pH 5.0 to 5.2, they may stop forming root nodules and thus inhibit the legume growth that otherwise could occur. Below pH 4.5, rhizobia stop multiplying. For these reasons, lime often is applied to acid soils to maintain a suitable environment for the *Rhizobium*-legume association. Acid fertilizers and those containing copper and zinc can kill rhizobia, and many other materials such as herbicides, seed fungicides, and insecticides can have deleterious effects on rhizobia. Not all rhizobia are susceptible to all the treatments indicated, but it is frequently difficult to predict the effect of any treatment on a specific pasture. If any such treatments are economically feasible and biologically required in a pasture environment, they should be used only after the *Rhizobium*-legume association has been established, not at the time of seeding.

Nitrogen fertilizers are particularly inhibitory to rhizobia. Even at very low levels, soluble nitrogen prevents root nodule formation. For that reason, one should never add nitrogen fertilizers at the time pelleted seeds are planted in a dryland pasture environment.

Molybdenum is required by nitrogen-fixing rhizobia. In most dryland pasture environments there is enough molybdenum in the soil for normal levels of forage production. However, when total forage production is increased by seeding

Table 4. Effectiveness of various inoculation methods in coating clover seeds with rhizobia

Treatment*	Seed condition	Bacteria† per seed
1	Uninoculated	0a
2	Inoculated by producer A	656,000b
3	Inoculated by producer B, process 1	2,600c
4	Inoculated by producer B, process 2	51,500d
5	UC method, producer C peat, normal product rate	925e
6	UC method, producer C peat, 2 × product rate	3,500f
7	UC method, producer C peat, 4 × product rate	35,000d
8	Producer C peat + producer C glue, normal product rate	6,000cf

* Seeds were sterilized with mercuric chloride before they were supplied to producers A and B for their proprietary coating procedures (treatments 2 to 4). Other sterile seeds from the same lot were inoculated with a commercial peat *Rhizobium* inoculant according to the method given under *Pellet Inoculation of Legume Seed*, except that the amount of peat varied from the product rate suggested on the package (treatment 5) to two times the amount (treatment 6) or four times the amount (treatment 7). Equivalent seeds were pelleted for treatment 8 according to label instructions sold with the commercial peat and glue product discussed under *Pellet Inoculation of Legume Seed*.

† Average of six replicate seed samples. Values followed by different letters are significantly different at $P < 0.05$. For each seed sample, 10 Woogenellup subterranean clover seeds were homogenized in a buffered solution within 48 hours of inoculating, and plated in serial dilutions on YEM bacterial medium. Apparent rhizobia colonies were counted 3 days later. Data in this table were collected by D. A. Phillips in cooperation with J. E. Street and B. L. Kay.

11

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842.*

Page 12

inoculated clovers and fertilizing with phosphorus and sulfur, a small amount of molybdenum fertilizer can provide an additional increment of forage production (Jones and Ruckman 1973). A slight excess of molybdenum in forage can be toxic to livestock, however, and fertilization with this element must be done with caution.

Soil factors critical for legumes

Rhizobia cannot promote growth of legumes by fixing nitrogen unless all other nutrients required by the legumes are available in the soil. Thus a rancher should carefully consider whether a lack of such important nutrients as phosphorus, sulfur, and potassium will limit plant growth once more nitrogen is available. Potassium deficiencies seldom occur in California (Jones 1974), but the importance of phosphorus and sulfur for maximizing yields in California dryland pasture environments is well established (Jones 1964, Jones and Ruckman 1973). Typical data for three soil types at separate Mendocino County sites are shown in table 5. Because it is difficult to predict which element will limit growth at any specific site, the safest course of action is to fertilize with 200 pounds per acre of 0-38-0-20, a mixture that provides both phosphorus and sulfur by combining treble superphosphate and elemental sulfur (Phillips, Jones, and Center 1987).

Nitrogen fertilizers generally decrease nitrogen fixation by favoring the growth of grass plants. Jones and Winans (1967) reported that the proportion of annual clover plants in a grazed pasture decreased from 35 percent to 11 percent of the total when nitrogen fertilizer was added at a rate of 80 pounds per acre. Nitrogen fertilizer is never recommended for a pasture containing legumes.

Proper grazing to maintain annual clovers

After an initial investment to plant an inoculated clover into a dryland pasture, proper grazing management and periodic applications of phosphorus and sulfur can maintain high production from the clover plants indefinitely. The key to this desirable end lies in appropriate grazing management. A properly grazed subterranean or rose clover pasture will have closely cropped plants during the growing season. A failure to provide adequate grazing pressure will favor growth of grasses and seriously reduce the proportion of annual clovers in a California dryland pasture (Jones and Winans 1967). Animals should be removed at flowering and seed set, but they must be returned during summer months to remove plant litter. Too much litter will favor germination of grasses the following fall.

Table 5. Effects of phosphorus (P) and sulfur (S) fertilizer on yield of dryland pastures improved with *Rhizobium*-inoculated subterranean clover*

Soil	Fertilizer	Forage dry matter†	Forage nitrogen
		lb/ac	*percentage*
Laughlin loam	None	2,900a	1.98a
	P	7,300b	2.29b
	S	4,700c	2.26a
	P+S	7,500b	2.55b
Yorkville clay	None	2,800a	2.32a
	P	3,200a	2.04a
	S	4,000b	2.48b
	P+S	4,200b	2.71b
Sutherlin loam	None	3,400a	2.19a
	P	6,200b	2.44a
	S	4,400a	2.59b
	P+S	6,100b	2.59b

* Native pastures on three sites in Mendocino County were sown with Woogenellup subterranean clover and Blando brome grass in October 1979. Clover seeds were pelleted with a commercial peat inoculant, and plots received 0 or 100 pounds per acre of P or S as indicated. Forage was harvested in April 1980. Data are from D. A. Phillips, M. B. Jones, and D. M. Center (1987).

† Average of four replicate 3- × -5-foot plots. Values followed by different letters are significantly different at $P < 0.05$ for that site.

12

Phillips, DA; Williams, WA. 1987. Range legume inoculation and nitrogen fixation by root-nodule bacteria. *University of California Agricultural Experiment Station Bulletin 1842.*

Page 13

LITERATURE CITED

Brockwell, J.
1962 Studies on seed pelleting as an aid to legume seed inoculation. 1. Coating materials, adhesives and methods of inoculation. *Aust. J. Agric. Res.* 13:638–49.
Brockwell, J., R. R. Gault, D. L. Chase, F. W. Hely, M. Zorin, and E. J. Corbin
1980 An appraisal of practical alternatives to legume seed inoculation: Field experiments on seed bed inoculation with solid and liquid inoculants. *Aust. J. Agric. Res.* 31:47–60.
Brockwell, J., D. F. Herridge, R. J. Roughley, J. A. Thompson, and R. R. Gault
1975 Studies on seed pelleting as an aid to legume seed inoculation. 4. Examination of preinoculated seed. *Aust. J. Exp. Agric. Anim. Husb.* 15:780–87.
Burns, R. C., and R. W. F. Hardy
1975 *Nitrogen fixation in bacteria and higher plants.* New York: Springer-Verlag. 189 pp.
Evans, H. J., P. J. Bottomley, and W. E. Newton, eds.
1985 *Nitrogen fixation research progress, Proceedings of the sixth international symposium on nitrogen fixation.* Corvallis, Oregon 97331, August 4–10, 1985. Dordrecht: Martinus Nijhoff Publ. 731 pp.
Jones, M. B.
1964 Effect of applied sulfur on yield and sulfur uptake of various California dryland pasture species. *Agron. J.* 56:235–37.
1974 Fertilization of annual grasslands of California and Oregon. In *Forage fertilization*, ed. D. Mays, 255–75. Madison: American Society of Soil Agronomy.
Jones, M. B., J. C. Burton, and C. E. Vaughn
1978 Role of inoculation in establishing subclover on California annual grasslands. *Agron. J.* 70:1081–85.
Jones, M. B., and J. E. Ruckman
1973 Long-term effects of phosphorus, sulfur, and molybdenum on a subterranean clover pasture. *Soil Sci.* 115:343–48.
Jones, M. B., and S. S. Winans
1967 Subterranean clover versus nitrogen fertilized annual grasslands: Botanical composition and protein content. *J. Range Manage.* 20:8–12.
Loneragan, J. F., D. Meyer, R. G. Fawcett, and A. J. Anderson
1955 Lime pelleted clover seeds for nodulation on acid soils. *J. Aust. Inst. Agric. Sci.* 21:264–65.
Martin, W. E., W. A. Williams, and W. H. Johnson
1957 Refertilization of rose clover. *Calif. Agric.* 11(12):7–9, 14.
Phillips, D. A., and J. P. Bennett
1978 Measuring symbiotic nitrogen fixation in rangeland plots of *Trifolium subterraneum* L. and *Bromus mollis* L. *Agron. J.* 70:671–74.
Phillips, D. A., M. W. Demment, and L. R. Teuber
1985 Breeding for increased forage yield and quality in alfalfa. In *Fifteenth California alfalfa symposium*, ed. V. L. Marble, 40–44. Davis: University of California Agronomy Extension.
Phillips, D. A., M. B. Jones, and D. M. Center
1987 Intensive management of small-scale rangeland units increases forage and lamb productivity. *Calif. Agric.* 41(1&2):4–5.
Williams, L. E., and D. A. Phillips
1983 Increased soybean productivity with a *Rhizobium japonicum* mutant. *Crop Sci.* 23:246–50.
Williams, W. A., J. V. Lenz, and A. H. Murphy
1954 Nature of beneficial effect of subterranean clover straw on establishment of subterranean clover. *Agron. J.* 46:95–96.
Williams, W. A., R. M. Love, and L. J. Berry
1957 Production of range clovers. *Calif. Agric. Exp. St. Circ.* 458. 19 pp. Out of print.

13

Section 3
Summary Review of Standard Specification 20-2.10

See **Section 4** for the Expanded Review of Standard Specification 20-2.10.

This section provides an expanded evaluation of the procedural execution of legume inoculation as detailed in *UC-AES Bulletin 1842*. At the end of this section, **Table 3-3** provides a summary of the factors that affect legume inoculation, the problems with the existing Caltrans legume inoculation protocol, and the options for revising the standard specifications to remedy these problems.

3.1 Intended Usage of *Bulletin 1842* Protocol

UC-AES Bulletin 1842 **is intended for range pasture legumes, not specifically highway revegetation with native legumes.**

As the title of *UC-AES Bulletin 1842* states, "Range-Legume Inoculation and Nitrogen Fixation by Root-Nodule Bacteria," the intended use of the legume inoculation protocol is by ***ranchers*** on ***rangeland pastures*** for ***livestock grazing***, not for revegetation of roadsides or wildlands. This repurposing for highway revegetation of an existing rangeland protocol has bearing on the differences in legume lifeforms used, the degree of rhizobium to legume species matching, the legume seed application method used, the legume seed application rate required, and the implicit vegetation succession model held. Legume inoculation for revegetation departs substantially from highly managed agricultural conditions [**65**]. These issues are compared in **Table 3-1**.

Table 3-1. Comparison of How Rangeland Protocol of *UC-AES Bulletin 1842* Applies to Roadsides.

Issue	Rangeland	Roadside
Legume Lifeforms	Alien Forbs	Native Forbs, SubShrubs, and Shrubs; Alien Forbs
Rhizobium-Legume Matching	High	Largely unknown, untested, and likely poor
Application Method	UC-AES 1842 designed for drill-seeding pelleted seed	Caltrans Contract Specs typically hydroseed pelleted-inoculated legumes
Seed Application Rate	UC-AES 1842 recommends final legume density of ≥ 20 plants per ft^2	Caltrans Contract Specs typically do not seed legumes at > 3 plants per ft^2
Implicit Succession Model	**Stasis**: indefinite maintenance of herbaceous legumes and grasses for grazing	**Facilitation**: shift over time from annual forbs and grasses to perennial forbs, grasses, and shrubs

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

1. Inoculated legume seeds can be obtained by one of three methods:

(A) by purchasing pre-inoculated seeds
(B) by contracting for custom inoculation services
(C) by the rancher pelleting seeds on-site.

Method A is not recommended because the shelf-life of rhizobia on inoculated seeds is very short.

Method B can be successful, but the supplier and the expenses must be investigated carefully.

This bulletin is directed toward helping individuals use Method C to pellet their own legume seeds.

3.2 Inoculation Provider

Caltrans recognizes three options for inoculation execution:

- **pre-inoculation** by commercial/industrial professionals;
- **custom inoculation** by the seed vendor or contractor;
- **ad hoc** inoculation by the revegetation landscape contractor.

Each option can be effective, but the set of potential problems associated with each factor in the inoculation process may not be equivalent among the options, e.g., a professional laboratory setting may have more precision equipment for effecting inoculation than the landscape contractor. Minimization of any of these problems is dependent upon the equipment and abilities of each individual provider.

Table 3-2 lists the potential problems by category, *references* the subsection which details the potential problems for that category, *indicates* the degree to which the Standard Specification 2002-2.10 currently addresses the problem, and *provides* a very generalized estimate of which provider option may offer the most effective problem mitigation, although this is prone to being highly variable.

Table 3-2. Synopsis of Potential Problems With Inoculation Factors Relative to Provider Category.

Problem Potential Among Providers : = nearly equivalent ▲ increases problem ▽ decreases problem

Sub-section	4.4	4.5	4.5	4.7	4.7	4.3	4.6	4.6	4.6
Factor	Inoculant Purity	Shelf Life	Storage Conditions	Post-Inoculation Seed Storage Time	Post-Inoculation Seed Storage Conditions	Strain Selection	Rate of Inoculation	Inoculation Equipment & Expertise	Inoculation Check
Factor Affects	Viability					Effectiveness			
Provider									
Pre-Inoculation	=	=	▽	▲	▽	=	▽	▽	▽
Custom Inoculation	=	=	=	=	▲	=	▽	▽	▲
Landscape Contractor	=	=	=	=	▲	=	▲	▲	▲
CT SPEC Control	No	Yes	No	Yes	No	Partial	Partial	Partial	No

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

2. Select a good commercial peat inoculant that contains root-nodule bacteria specific for the legume to be planted. Make sure the legume species is named on the label. No other inoculant is suitable.

Note

UC-AES Bulletin 1842 (see **Box 2.4**, number 14) recommends evaluating inoculation success by extracting legume seedlings to look for pinkish nodules on developing roots. This method can be inadequate because the same rhizobia species can interact with the same host as:

- a **mutualist** (N_2-fixing for the host),
- a **non-symbiont** unable to infect and nodulate with the host, *or*
- a **parasite** that infects the host but does fix significant amounts of N_2.

3.3 Selection of Rhizobial Strains

Rhizobial bacteria exhibit varying degrees of specificity and fidelity to individual legume host plant. Thus, correct association of rhizobia to legume species used is a minimum requirement for proper inoculation.

Problem 3.3.1 ► Lack of Standards

- **Lack of specificity about degree of compatibility or effectiveness of N_2-fixation**
- **Lack of industry-wide standards regarding compatibility or effectiveness**
- **Strains vary among manufacturers**

Rhizobial strains compatible with particular legumes may successfully form partnerships, but the rhizobium may not necessarily be efficient at N_2-fixation under roadside conditions. It is well-documented that effectiveness of N_2-fixation is highly variable among strains that will cause nodulation and this is especially true under field conditions where there may be indigenous rhizobia already present. Strains vary among manufacturers and there are no National, State or industry-wide compliance standards to which any product can be held accountable.

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

Pre-test legumes for each project to demonstrate and quantify the effectiveness of the strains being used under particular roadside conditions.

Problem 3.3.2 ► Lack of Guidance

- ***UC-AES Bulletin 1842* lacks guidance regarding rhizobial strains for the native legumes often called for in Caltrans revegetation seed mixes.**

  Most research regarding symbiotic N_2-fixation has been carried out within an agricultural context. Hence, there is scant information on wildland rhizobia and the ecological controls of symbiotic N_2-fixation in natural ecosystems. Rhizobia that are compatible and effective with agricultural legumes may not be effective with California native legumes.

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

Provide for field recovery and identification of nodule occupants of native legumes. As *UC-AES Bulletin 1842* recommends, strains of rhizobia to be used as inoculants can be isolated from nodules on vigorous legumes. These strains should be selected from the general geographic region in which the inoculants are to be used, then tested under controlled conditions.

BULLETIN 1842

SEED INOCULATION AND FIELD PROBLEMS

The *UC-AES Bulletin 1842* recommends contacting the local UC Extension Farm Advisor Office for manufacturer information on inoculant purity.

3.4 Inoculant Purity

Inoculant cultures manufactured in North America are noted to suffer from both poor numerical quality and questionable purity. Reportedly there are substantial differences among commercial inoculants and quality control methods. Many have been shown to be unsatisfactory due to contamination.

Problem 3.4.1 ► Lack of Purity Standards

- **Lack of industry-wide standards regarding purity**
- **Purity varies among manufacturers**

S P E C I F I C A T I O N   R E V I S I O N

There is no simple way for Caltrans to modify the specification.

Options

- Require a purity test to accompany every inoculant purchase.
- Work with manufacturers to improve and ensure purity standards.

The *UC-AES Bulletin 1842* recommends contacting the local UC Extension Farm Advisor Office for manufacturer information.

BULLETIN 1842

SEED INOCULATION AND FIELD PROBLEMS

4. Check the freshness of the inoculant by referring to the expiration date printed on the container. The inoculant is a living culture of root nodule bacteria that can be killed by drying and by high temperatures. Make sure the culture has been stored under refrigeration. Poor storage conditions can cause nodulation failure by reducing the number of viable bacteria in the inoculant.

3.5 Inoculant Shelf Life and Storage Conditions

Viability is monitored solely by the expiration date (shelf life) as determined by the manufacturer. Commercial cultured rhizobial inoculants are living preparations with limited shelf lives. The number of living rhizobia per package decline quickly over time [**33**]. Contaminants within the inoculum also decrease the survival of rhizobia and shorten the shelf life [**45**]. To slow the rate of loss, the quality of the storage conditions must be closely monitored; usually this requires refrigeration at an optimum temperature as indicated on the package by the manufacturer.

Problem 3.5.1 ► Expiration Date

Standard Spec 2002-20-2.10 requires only the date of inoculation to be shown on the package, however *UC-AES Bulletin 1842* states that the expiration date on the inoculant container should be observed.

Options

SPECIFICATION REVISION

- Revise the Standard Specifications to require monitoring both the expiration date provided on the package as well as the date of inoculation.
- Should the expiration date occur before the inoculation date, the inoculated seeds should be rejected by the Project Engineer.
- Should the expiration date occur before the scheduled project seed application date, the inoculated seeds should be rejected by the Project Engineer.

The *UC-AES Bulletin 1842* recommends contacting the local UC Extension Farm Advisor Office for manufacturer information.

Problem 3.5.2 ► Inoculant Storage

There are no requirements to ensure the inoculant has been stored appropriately so that the bacteria meet a quantitative minimum standard of viability.

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

Require a quantitative test of minimum viability standards performed by a laboratory immediately prior to the Project Engineer accepting the product from the landscape contractor.

BULLETIN 1842

SEED INOCULATION AND FIELD PROBLEMS

4. Use the inoculant at the rate the manufacturer recommends on the package, or, even better, at four times the recommended rate. Never use a lower amount, or there will be too few bacteria on each seed to produce good nodulation.

UC-AES Bulletin 1842 **provides no guidance for inoculation of native legumes.**

3.6 Rate of Inoculation

The rate of inoculation, i.e., the minimum number of rhizobial inoculants per seed, is critical to improve the probability of nodulation. In general, when the number of viable rhizobia inoculated per seed increases, nodulation is improved [**33**]. Exact numbers required are not definable, but in this case, the axiom "more is better" is true. Estimates of optimum numbers of rhizobia per seed are extremely variable, attaining up to 1 million for large-sized legume seeds.

UC-AES Bulletin 1842 cites 1,000 rhizobia per seed as a minimum and provides a comparison of inoculation methods with results ranging from 925-656,000 bacteria per seed. To achieve this it recommends using 4 times the suggested application rate listed on the inoculant package. Caltrans requires legume seeds to be pelleted at a rate of 2kg (2lbs) inoculum/100kg (100lbs) legume seed. Individual project specifications [raw data from **34**] confirm project specification instructions of this minimum and in some situations the minimum rate has been increased from 2 to 5 times the suggested application rate.

Problem 3.6.1 ► No Effectiveness Monitoring

- **Independent evaluations of inoculant products have shown that with a lack of industry-wide standards it is likely that suboptimal rates of inoculation can occur even with appropriate application rates.**
- **There is no monitoring to quantify the effectiveness of the inoculation, i.e., the average number of rhizobia that adhered to seeds for any given project.**
- **Thus, there is no mechanism by which a contractor or seed vendor to know if their inoculation methods have been successful or whether the product met target levels.**

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

Require testing of seed inoculation product by a contracted laboratory to evaluate the average number of rhizobia per seed for each legume species inoculated.

The *UC-AES Bulletin 1842* under sidebar "Pellet Inoculation of Legume Seed" provides quantification for differential amounts of seed, adhesive, and calcium carbonate to be used with the commonly cultivated rangeland legumes, clovers, vetch and alfalfa, using seed size as the discriminating factor (**Box 2.2**). There may be additional quantitative instructions on inoculant packaging.

Problem 3.6.2 ► Native Legumes

- ***UC-AES Bulletin 1842* provides no guidance regarding seed size or mixture quantities for the California native legumes often specified in roadside seed mixes.**
- **Inoculant packaging with quantitative instructions may be too general for species complexes with substantial variation in seed size; agricultural species may differ in seed size from native wildland species resulting in an inadequate rate of inoculation.**

Options

S P E C I F I C A T I O N R E V I S I O N

To standardize methods and improve inoculation rates Caltrans could supplement the *UC-AES Bulletin 1842* list and provide the quantitative data for all species of legumes called for in a seed mix, including native legumes.

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

6. Hold the inoculated seed in a cool, shady place, and plant as soon as possible into a seedbed that will receive a germinating rain in the very near future. If possible, plant into a moist seedbed after the first fall rain. Never plant in the summer months. Always remember that drying kills bacteria in the inoculant and reduces nodulation.

3.7 Post-Inoculation Seed Storage

Post-inoculation storage duration limitations are stated in the standard specification. Individual project specifications [**34**] have often decreased the maximum storage time from 90 to 30 days. Death of rhizobia on post-inoculated seeds is rapid; under conditions of heat and dessication a 95% mortality rate can occur within the first four hours after inoculation.

Problem 3.7.1 ► Time Limit

The 90-day post-inoculation storage period currently allowed by SS 20-2.10 is far too long. It may accommodate the business practices of industrial/commercial providers that inoculate large batches of seed in advance of specific orders, but it is unnecessary for the custom inoculation method or the landscape contractor inoculation method. It results in a significant reduction in rhizobial viability.

Options

SPECIFICATION REVISION

The Standard Specification should segregate the time limits for post-inoculation storage based upon the inoculation provider. The commercial/industrial provider may require a 90-day window; however, the custom inoculation and the landscape contractor inoculation should be reduced to 7-10 days.

Problem 3.7.2 ► Storage Conditions

Given the potential for complete mortality of rhizobia caused by high temperatures and desiccation during post-inoculation storage, the lack of guidance regarding this stage is unfortunate. *UC-AES Bulletin 1842*, written specifically for a rancher presumably without refrigeration capacity in the field away from the ranch facilities, suggests a shady cool storage location.

Options

SPECIFICATION REVISION

The Standard Specification should better define the post-inoculation storage conditions regarding temperature and humidity in order to keep the rhizobia alive.

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

***UC-AES Bulletin 1842* refers only to pelleted drill-seeding, not hydroseeding.**

3.8 Inoculated Seed Application Method

Seed application on slopes is usually done hydraulically. Hydroseeding combines pelleted legume seed, fertilizer, tackifier and wood fiber mulch into a water-based slurry that is then sprayed onto the roadside slopes in a single operation. This method has some potential to damage seed and legume inoculant from:

1) extended immersion in the hydroseeder tank solution,
2) acidity of the solution caused by fertilizers, and
3) physical dislodgement of the inoculant from the seed during agitation and application processes.

***UC-AES Bulletin 1842* refers only to pelleted drill-seeding.** This Bulletin was assembled for the purpose of guiding rangeland N-augmentation via inoculated legumes and, as such, is not completely applicable to the Caltrans roadside conditions.

Problem 3.8.1 ► Hydroseeding

Hydroseeding has the potential to damage or dislodge the inoculant from the seed. Some Caltrans project specifications have required the inoculated legume seed to be dry broadcast separately from the hydroseed application [as compiled in 34], but no follow-up monitoring was available to evaluate the effectiveness of that approach.

Options

SPECIFICATION REVISION

Dry broadcasting inoculated legume seed separately from the hydroseed application would likely improve the numbers of viable rhizobia per seed. The sequence of this two-step application would depend upon the germination characteristics of the legumes being used, mostly optimum burial depth, and the thickness and composition of the hydroseed mulch mix.

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

UC-AES Bulletin 1842 **provides no guidance for native legume seeding rates.**

A density of 20 plants per square foot equals how many plants per hectare?

20 plants	43560 ft^2	2.47 acre	= **2151864 plants**
ft^2	acre	ha	**ha**

3.9 Inoculated Seed Application Rate

The rate of seeding inoculated legumes on roadside revegetation projects is not indicated by the Standard Specifications, but left to the discretion of the project landscape architect. Rangeland seeding rates recommended by *UC-AES Bulletin 1842* specify a legume density ≥ 20 plants per ft^2 (≥ 871,200 per acre or 2,152,000 per ha).

Agricultural crop monocultures are grown at densities exceeding 1,000,000 plants/ha to achieve 100kg N/ha/yr. The use of a monoculture may not satisfy the roadside revegetation goals of Caltrans which have been multi-species oriented. A high density of legume plantings is necessary to both maximize the potential amount of N_2 fixed and to take advantage of any N-transfer that can occur from legumes to non-legumes in close proximity. Because N-transfer from legumes to non-legumes is highly localized, a high density of legumes is required to effect a significant augmentation to the N pool throughout the roadside area.

Rate of seeding for legumes used in roadside revegetation across the state has been highly variable. Caltrans project seeding rates for legumes are highly variable, but typically do not exceed 3 plants per ft^2, and are frequently 1 or less per ft^2.

Problem 3.9.1 ► Seed Application Rate Guidelines

While the inoculated legume seeding rate would be expected to vary among project sites, there are currently no guidelines for the seeding rates that correspond to levels of N (short- to long-term), soil organic matter, and sustainable N-cycling.

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

Options

Develop a research program to better correlate the levels of soil N (short- to long-term), soil organic matter, and N-cycling with inoculated legume seeding rates to achieve target N-augmentation levels.

3.10 Site Physical Conditions

Many physical factors of the site strongly influence the success of effective nodulation and N_2-fixation [**104**]. Negative conditions include very high or low soil temperatures, low soil moisture, extremes of soil pH, soil fertility levels, chemical seed treatments, chemical fertilization, and the presence of indigenous or naturalized rhizobia can suppress legume nodulation by cultured inocula [**66**,**103**,**132**,**133**,**134**].

UC-AES Bulletin 1842 **outlines six instructional guidelines regarding site physical conditions (see sidebar). None of these factors are addressed directly in the Standard Specification.**

Problem 3.10.1 ► Information Standardization and Access

- **The information contained in *UC-AES Bulletin 1842* is not presented in an efficient format for individual Caltrans projects.**
- **Some of the guidelines have been ignored or overlooked as evidenced from individual project specifications [compiled in 34]; e.g., many projects require both inoculated legumes in the seed mix and nitrogen fertilization that will serve to suppress N_2-fixation.**
- **In addition, not all of the information presented in the Bulletin is written for the Caltrans context; the rancher/ rangeland perspective does vary in many aspects from roadside revegetation.**

Options

S P E C I F I C A T I O N   R E V I S I O N

There is no simple way for Caltrans to modify the specification.

All appropriate staff should be issued copies of *UC-AES Bulletin 1842*, however, because it is not completely applicable to the Caltrans context, it should be condensed and reformatted to more briefly define and standardize the ancillary requirements necessary when legume inoculation is chosen as an N-augmentation option.

BULLETIN 1842

SEED INOCULATION AND FIELD PROBLEMS

7. Do not mix acid fertilizers with inoculated seeds, and do not sow seeds in contact with such fertilizers. The acidity may kill most or all of the root-nodule bacteria.

8. For the same reason, do not mix the seeds with fertilizers that contain trace elements, unless the manufacturer has specifically formulated and recommended the fertilizer product for that use.

9. Do not use herbicides, fungicides. or any other pesticides when planting inoculated seeds. Many of these poisons are highly toxic to root-nodule bacteria.

10. Check the acidity of the soil where inoculated seeds will be planted. Legumes usually fail to nodulate when the soil is more acid (lower pH) than pH 5.2. If the soil pH is too low. an appropriate amount of lime should be added to the soil at the time of planting. Drilling lime in with the seed is suitable, but coating seeds with calcium carbonate accomplishes the same thing and is compatible with broadcasting the seeds.

11. Make sure the soil contains adequate amounts of plant nutrients. A legume suffering from a deficiency of any nutrient other than nitrogen cannot benefit fully from inoculation. To maximize grazing potentials the legumes must have an adequate supply of available phosphorus and sulfur, the elements most commonly deficient on California rangelands. Many pasture legumes such as subterranean clover are less competitive than grasses for soil nutrients.

12. Do not use nitrogen fertilizers when planting legumes in a pasture. Well-nodulated legumes do not need any nitrogen from the soil, and nitrogenous fertilizers usually give grasses a competitive advantage over legumes. Moreover, ammonia, nitrates, and nitrites inhibit legume nodulation under most circumstances.

Problem 3.10.2 ► Lack of Site Soil Data

- **There are no requirements to quantify soil fertility or pH in order to ascertain the need for legume inoculation or chemical fertilization.**
- **There are no specifications regarding testing for indigenous rhizobia that may suppress the inoculated rhizobia. Landscape architects must make decisions regarding environmental factors in the absence of data.**

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

Landscape architects must be able to make N-augmentation decisions based on data. Each project should require a data acquisition protocol for the post-construction roadside including: soil fertility, soil pH, % organic matter, and the presence of indigenous or naturalized rhizobia.

Problem 3.10.3 ► Season of Seed Application

- **High and low soil temperatures can kill any cultured rhizobia that have survived the inoculation and seed application procedures. Desiccation in a dry seed bed can do the same.**
- ***UC-AES Bulletin 1842*** **recommends that inoculated seed be planted into a moist seedbed that will receive a germinating rain soon thereafter.**
- **There are no specifications restricting the seasonal timing of seed application. Seeding during the hot and dry months of late spring, summer, or early fall will likely kill all inoculant.**
- **The landscape contractor must comply with contractual time limits that are beyond the control of the seed specification designer and do not consider climatic or biological time constraints.**

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

The *UC-AES Bulletin 1842* should be adhered to with regard to timing of seed application upon a moist seedbed. Revegetation contracts with inoculated legumes should restrict seed application time frames to seasonal climatic window appropriate to the site.

Table 3-3. Inoculation Factors: Summary of Problems and Specification Revision Options.

Inoculation Factor	Problem	Specification Revision Option
3.3 Selection of Rhizobial Strains	• Lack of specificity about degree of compatibility or effectiveness of N_2-fixation • Lack of industry-wide standards regarding compatibility or effectiveness • Strains vary among manufacturers • *UC-AES Bulletin 1842* lacks guidance regarding rhizobial strains for the native legumes often called for in Caltrans revegetation seed mixes.	**• Pre-test legumes for each project to demonstrate and quantify the effectiveness of the strains being used under roadside conditions.** **• Pre-test legumes for each project to demonstrate and quantify the effectiveness of the strains being used under roadside conditions.** **• Provide for field recovery and identification of nodule occupants of native legumes. As *UC-AES Bulletin 1842* recommends, strains of rhizobia to be used as inoculants can be isolated from nodules on vigorous legumes. These strains should be selected from the general geographic region in which the inoculants are to be used, then tested under controlled conditions.**
3.4 Inoculant Purity	• Lack of industry-wide standards regarding purity • Purity varies among manufacturers	**• Require a purity test to accompany every inoculant purchase.** **• Work with manufacturers to improve and ensure purity standards.** **The *UC-AES Bulletin 1842* recommends contacting the local UC Extension Farm Advisor Office for manufacturer information.**
3.5 Inoculant Shelf Life and Storage Conditions	• Standard Spec 2002-20-2.10 requires only the date of inoculation to be shown on the package, however *UC-AES Bulletin 1842* states that the expiration date on the inoculant container should be observed. • There are no requirements to ensure the inoculant has been stored appropriately so that the bacteria meet a quantitative minimum standard of viability.	**• Revise the Standard Specifications to require monitoring both the expiration date provided on the package as well as the date of inoculation. Should the expiration date occur before the inoculation date, the inoculated seeds should be rejected by the Project Engineer. Should the expiration date occur before the scheduled project seed application date, the inoculated seeds should be rejected by the Project Engineer. The culture expiration date must be adhered to for both seed inoculation and roadside seed application time frames.** **• Require a quantitative test of minimum viability standards performed by a laboratory immediately prior to the Project Engineer accepting the product from the landscape contractor.**
3.6 Rate of Inoculation	• Independent evaluations of inoculant products have shown that with a lack of industry-wide standards it is likely that suboptimal rates of inoculation can occur even with appropriate application rates. • There is no monitoring to quantify the effectiveness of the inoculation, i.e., the average number of rhizobia that adhered to seeds for any given project. • Thus, there is no mechanism by which a contractor or seed vendor to know if their inoculation methods have been successful or whether the product met minimum levels. • *UC-AES Bulletin 1842* provides no guidance regarding seed size or mixture quantities for the California native legumes often specified in roadside seed mixes. • Inoculant packaging with quantitative instructions may be too general for species complexes with substantial variation in seed size; agricultural species may differ in seed size from native wildland species resulting in an inadequate rate of inoculation.	**• Require testing of seed inoculation product by a contracted laboratory to evaluate the average number of rhizobia per seed for each legume species inoculated.** **• To standardize methods and improve inoculation rates Caltrans could supplement the *UC-AES Bulletin 1842* list and provide the quantitative data for all species of legumes called for in a seed mix, including native legumes.**

Table 3-3. (cont.)

Inoculation Factor	Problem	Specification Revision Option
3.7 Post-Inoculation Seed Storage	• The 90-day post-inoculation storage period currently allowed by SS 20-2.10 is far too long. It may accommodate the business practices of industrial/ commercial providers that inoculate large batches of seed in advance of specific orders, but it is unnecessary for the custom inoculation method or the landscape contractor inoculation method. It results in a significant reduction in rhizobial viability. • Given the potential for complete mortality of rhizobia caused by high temperatures and dessication during post-inoculation storage, the lack of guidance regarding this stage is unfortunate. *UC-AES Bulletin 1842*, written specifically for a rancher presumably without refrigeration capacity in the field away from the ranch facilities, suggests a shady cool storage location.	**• The Standard Specification should segregate the time limits for post-inoculation storage based upon the inoculation provider. The commercial/ industrial provider may require a 90-day window, however, the custom inoculation and the landscape contractor inoculation should be reduced to 7-10 days.** **• The Standard Specification should better define the post-inoculation storage conditions regarding temperature (optimally 5°C) and humidity in order to keep the rhizobia alive.**
3.8 Inoculated Seed Application Method	• Hydroseeding has the potential to damage or dislodge the inoculant from the seed. Some Caltrans project specifications have required the inoculated legume seed to be dry broadcast separately from the hydroseed application, but no follow-up monitoring was available to evaluate the effectiveness of that approach.	**• Dry broadcasting inoculated legume seed separately from the hydroseed application would improve the numbers of viable rhizobia per seed. The sequence of this two-step application would depend upon the germination characteristics of the legumes being used, mostly burial depth, and the thickness and composition of the hydroseed mulch mix.**
3.9 Inoculated Seed Application Rate	• While the inoculated legume seeding rate would be expected to vary among project sites, there are currently no guidelines for the suggested seeding rates that correspond to the fertility levels of the site or other environmental conditions.	**• Develop a research program to better correlate the level of roadside soil fertility with inoculated legume seeding rates to achieve target N levels.**
3.10 Site Physical Conditions	• The information contained in *UC-AES Bulletin 1842* is not presented in an efficient format for individual Caltrans projects. • Some of the guidelines have been ignored or overlooked as evidenced from the specifications included in the report by CH2MHILL [**34**]; e.g., many projects require both inoculated legumes in the seed mix and nitrogen fertilization that will serve to suppress N_2-fixation. • In addition, not all of the information presented in the Bulletin is written for the Caltrans context; the rancher/rangeland perspective does vary in many aspects from roadside revegetation, leading to potential confusion. • There are no requirements to quantify soil fertility or pH in order to ascertain the need for legume inoculation or chemical fertilization. • There are no specifications regarding testing for indigenous rhizobia that may suppress the inoculated rhizobia. Landscape architects must make decisions regarding environmental factors in the absence of data. • High and low soil temperatures can kill any cultured rhizobia that have survived the inoculation and seed application procedures. Desiccation in a dry seed bed can do the same. • *UC-AES Bulletin 1842* recommends that inoculated seed be planted into a moist seedbed that will receive a germinating rain soon thereafter. • There are no specifications restricting the seasonal timing of seed application. Seeding during the hot and dry months of late spring, summer, or early fall will likely kill all inoculant. • The landscape contractor must comply with contractual time limits that are beyond the control of the seed specification designer and do not consider climatic or biological time constraints.	**• All appropriate staff should be issued copies of *UC-AES Bulletin 1842*, however, because it is not completely applicable to the Caltrans context, it should be condensed and reformatted to better and more briefly define and standardize the ancillary requirements necessary when legume inoculation is chosen as an option.** **• Landscape architects must be able to make N-augmentation decisions based upon data. Each project requires a data acquisition agenda for the post-construction roadside including: soil fertility, soil pH, % organic matter, and the abundance of indigenous or naturalized rhizobia.** **• *UC-AES Bulletin 1842* should be adhered to with regard to timing of seed application. Revegetation contracts with inoculated legumes should restrict seed application time frames to seasonal climatic window appropriate to the site.**

Section 4
Expanded Review of Standard Specification 20-2.10

See Section 3 for the Summary Review of Standard Specification 20-2.10.

This section provides an expanded evaluation of the procedural execution of legume inoculation as detailed in *UC-AES Bulletin 1842*. See **Table 3-3** at the end of **Section 3** for a summary of the factors that affect legume inoculation, the problems with the existing Caltrans legume inoculation protocol, and the options for revising the standard specifications to remedy these problems.

4.1 Intended Usage of *Bulletin 1842* Protocol

***UC-AES Bulletin 1842* is intended for range pasture legumes, not specifically highway revegetation with native legumes.**

As the title of *UC-AES Bulletin 1842* states, "Range-Legume Inoculation and Nitrogen Fixation by Root-Nodule Bacteria," the intended use of the legume inoculation protocol is by ***ranchers*** on ***rangeland pastures*** for ***livestock grazing***, not for revegetation of roadsides or wildlands. This repurposing for highway revegetation of an existing rangeland protocol has bearing on the differences in legume lifeforms used, the degree of rhizobium to legume species matching, the legume seed application method used, the legume seed application rate required, and the implicit vegetation succession model held. Legume inoculation for revegetation departs substantially from highly managed agricultural conditions [**65**]. These issues are compared in **Table 4-1**.

Table 4-1. Comparison of How Rangeland Protocol of *UC-AES Bulletin 1842* Applies to Roadsides.

Issue	Rangeland	Roadside
Legume Lifeforms	Alien Forbs	Native Forbs, SubShrubs, and Shrubs; Alien Forbs
Rhizobium-Legume Matching	High	Largely unknown, untested, and likely poor
Application Method	UC-AES 1842 designed for drill-seeding pelleted seed	Caltrans Contract Specs typically hydroseed pelleted-inoculated legumes
Seed Application Rate	UC-AES 1842 recommends final legume density of ≥ 20 plants per ft^2	Caltrans Contract Specs typically do not seed legumes at > 3 plants per ft^2
Implicit Succession Model	**Stasis**: indefinite maintenance of herbaceous legumes and grasses for grazing	**Facilitation**: shift over time from annual forbs and grasses to perennial forbs, grasses, and shrubs

This review addresses legume seed inoculation for typical post-construction revegetation projects only.

Of 57 Caltrans roadside revegetation failures, about 30% exhibited "low soil fertility" even after a decade.

Following is a synopsis of the primary roadside context factors affecting legume inoculation:

1) road construction impacts on soils and soil nitrogen;

2) major pathways of nitrogen input and loss; and

3) revegetation models of Caltrans.

4.1.1 Road Construction Affects On Soils

Road construction activity significantly alters the existing plant growing conditions on roadsides, usually resulting severe disturbance to soils. Removal and redistribution of soil horizons as part of topographic reconfiguration brings about changes to physical, chemical and biological properties of soil. Typically topsoil is buried exposing subsoil and parent material. Where severe damage has occurred, in which original soils are lost and subsoils are exposed, nutrient poor horizons will not support plant growth [**20**]. Removal of all biological constituents, vegetation and soil biota in soil organic matter, all but arrests those biotically-mediated processes critical to the ecological functioning of terrestrial ecosystems: soil formation, nutrient cycling, energy transfers, plant re-establishment, and long-term sustainability [**110**].

Disruption of processes predicated on soil organic matter, critically impedes the rate at which disturbed ecosystems can begin recovery towards sustainable biological productivity [**96**,**141**]. Thus, post-construction roadsides can present both immediate and protracted hostile plant growth environments. Of 57 Caltrans roadside revegetation failures, some 30% presented "low soil fertility" even after a decade [**34**].

4.1.2 Road Construction Affects On Nitrogen

Nitrogen is the key nutrient in plant growth [**106**] and the nutrient that plants require in the largest amounts. In revegetation efforts this can be problematic because, after water availability, N is generally considered to be the most limiting plant growth factor in arid and semi-arid ecosystems [**15**].

Soil N occurs in both inorganic and organic forms, with the cycling between the two mediated by soil biota [**173**]. Soil disturbance, especially topsoil removal, disrupts this N cycling [**48**,**55**]. Thus, the removal of topsoil results in both immediate and protracted severe N deficiencies because soil organic matter in topsoil is the main storage reservoir for terrestrial N [**20**], holding more than 95% of soil N [**73**,**106**]. Road excavations have reduced total N from 650mg N/kg soil in topsoils to <200mg N/kg soil in underlying parent material [**39**].

When plant and microbial uptake of N are reduced, mineralized N is not cycled rapidly into the organic storage reservoir, leaving it vulnerable to hydrologic and atmospheric losses [110]. This exacerbates already low levels of N bioavailability [163], making successful revegetation unattainable for several decades. Total soil N and percentage organic matter are lower on filled-excavated sites, than on undisturbed sites even 7 decades after disturbance [38]. Other severe construction disturbance sites have documented lower levels of inorganic N availability versus predisturbance levels over similar timespans [43,144]. In North Dakota rates of N accumulation at strip-mined sites were calculated to require over 200 years to achieve the ecological equivalence to undisturbed sites [167].

Depletion of bioavailable N is often the limiting factor in revegetation [111,154]. Where topsoil has been lost, it has been necessary to apply at least 200kg/ha of a 20-10-10 NPK fertilizer as an immediate, but short-term, remedy for severe nutrient deficiencies [20]. Without remediation of N-cycling further soluble inorganic additions are commonly required or growth collapses [12,20]. Mineralizable N, organic yet decomposable, needs to provide 30-70kg N/ha/yr into the system to support annual plant growth [37]. While decomposition of organic matter is a major source of plant available N, notably, it is a very conservative process; organic matter contains about 5% by weight of N with only 1-3% of that released annually via decomposition [106]. To maintain long-term sustainable plant growth for disturbed soils, threshold N values stored in soil organic matter are estimated to fall between 1000kg total N/ha [20] and 1500kg total N/ha [37].

4.1.3 Nitrogen Dynamics In A California Annual Grassland

Substantial lengths of Caltrans roadsides are vegetated by non-native annual plants, usually by the same grasses that constitute the so-called California annual grassland. As such, the N dynamics of these ecosystems hold particular relevance to the N status along Caltrans roadsides located in low to moderate elevations of the mediterranean-type climate regions of the state. The dynamics of this system, summarized from Jackson et al. [85], demonstrate the relationship between N cycling and sustainable plant growth.

4.1.3.1 Spatial Nitrogen Distribution.

The majority of N occurs as soil organic N in the form of soil microbial biomass, with lesser amounts as plants and plant litter. N is primarily concentrated in the top 10cm of soil with total organic N greatest between 0-4cm.

4.1.3.2 Temporal Nitrogen Dynamics.

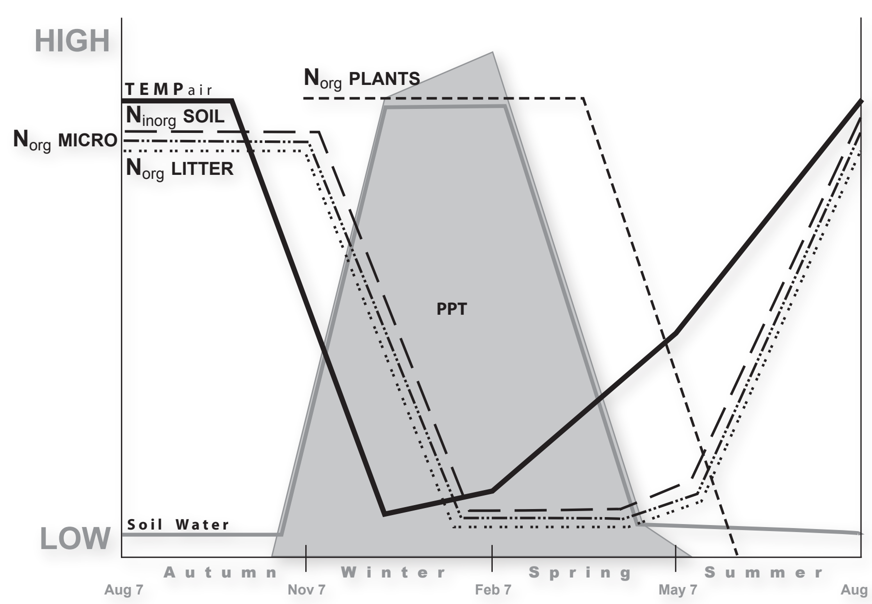
A tight link exists between seasonality of the mediterranean-type climate, annual plant life cycle and N dynamics (**Figure 4-1**). Autumn rains trigger the cycle: Plant seeds germinate, grow and absorb inorganic N from the soil reservoir. Organic N in microbiota and soil inorganic N decline as N is taken up by plants. With the end of spring rains plants senesce and die; during summer their tissues become plant litter and are decomposed by soil biota and organic N is transformed to soil inorganic N. Because soil inorganic N is water soluble it is vulnerable to loss by leaching during the first fall rain event.

4.1.4 Native Soils: Are California Soils N Deficient?

Native ecosystems in arid and semi-arid regions are usually poor in available N [**172**]. Conventional generalizations have characterized most of California's ecosystems as having N-deficient soils, including: forests [see **1**,**161**], chaparral [**70**,**76**], coastal sage scrub [**76**], and deserts [**31**,**170**]. Bear in mind that local soil N levels in proximity to Caltrans roadsides likely constitute an intricate spatiotemporal pattern that is far from homogeneous given the complex climatic, geologic, biologic and soil attributes throughout the state. Also, chronic air pollution is now recognized as a contributor to soil N levels in certain regions of the state. Thus, depending upon the particular environmental conditions of a region, some soils could be classified as being N-deficient, some have likely benefited from low

Figure 4-1. Seasonal Relationship Between Climate and N Dynamics in California Annual Grasslands.

[Adapted From **85** to show trends; not to scale].



to moderate anthropogenic inputs of nitrogenous depositions [29], and some with high levels of chronic N deposition are considered N-saturated [29].

4.1.5 Nitrogen Inputs to Terrestrial Systems

Two major pathways of N input to terrestrial ecosystems occur: atmospheric deposition and biological fixation [17,74]. **Figure 4-2** and **Figure 4-3** illustrate the basic nitrogen cycle.

4.1.5.1 Atmospheric Nitrogen Deposition.

Terrestrial N is derived principally from the earth's atmosphere where it constitutes some 78% of the components [122] and only somewhat from rocks from which it is released through weathering and soil formation [9]. A variety of forms of nitrogen exist in the atmosphere (**Table 4-2**), only some of which can be rapidly incorporated into terrestrial N after deposition [17].

The limitations of N for supporting plant growth are not manifest from an absence in the biosphere, but from being present in a chemical form that plants are unable to absorb. N is available for plant uptake in only two forms: ammonium (NO_3^-) and nitrate (NH_4^+). However, only a small portion of soil N is available in these inorganic forms at any one time [106].

Some regions of the state are experiencing noteworthy increases in soil N levels from atmospheric deposition. Sources are anthropogenic, associated with agricultural, industrial, and urban emissions [58]. Both emissions source and regional meteorology determine the transport and deposition pattern of the nitrogenous compounds [14]. The affected areas are generally sites downwind of large urban or agricultural areas [58]. The best documented regions include: 1) the Los Angeles Air Basin with the Transverse Ranges located downwind, and 2) the San Joaquin Air Basin with the western slopes of the southern Sierra Nevada Range located

Table 4-2. Major Forms of Atmospheric Nitrogen Deposition.
[From **18**].

Compound		Form
NO_3^-	Nitrate	Dissolved and aerosol
NH_4^+	Ammonium	Dissolved and aerosol
NO	Nitric Oxide	Gas
NO_2	Nitrogen Dioxide	Gas
HNO_3	Nitric Acid	Gas
NH_3	Ammonia	Gas

Nitrogen Cycle

Step	Process
1	N in plant animal residues and N derived from the atmosphere through electrical, combustion, and industrial processes (N_2 is combined with H_2 or 0_2) is added to the soil.
2	Organic N in the residues is mineralized to NH_4^+ by soil organisms. Plant roots absorb a portion of the NH_4^+.
3	Much of the NH_4^+ is converted to NO_3^- by nitrifying bacteria through nitrification.
4	NO_3^- and NH_4^+ are taken up by plant roots and used to produce the protein in plants.
5	Some NO_3^- is lost to groundwater systems as a result of downward movement through soil in percolating water.
6	Some NO_3^- is converted by denitrifying bacteria in N_2 and nitrogen oxides (N_2O or NO) that escape into the atmosphere, completing the cycle.
7	NH_4^+ can be converted to NH_3 through volatilization.

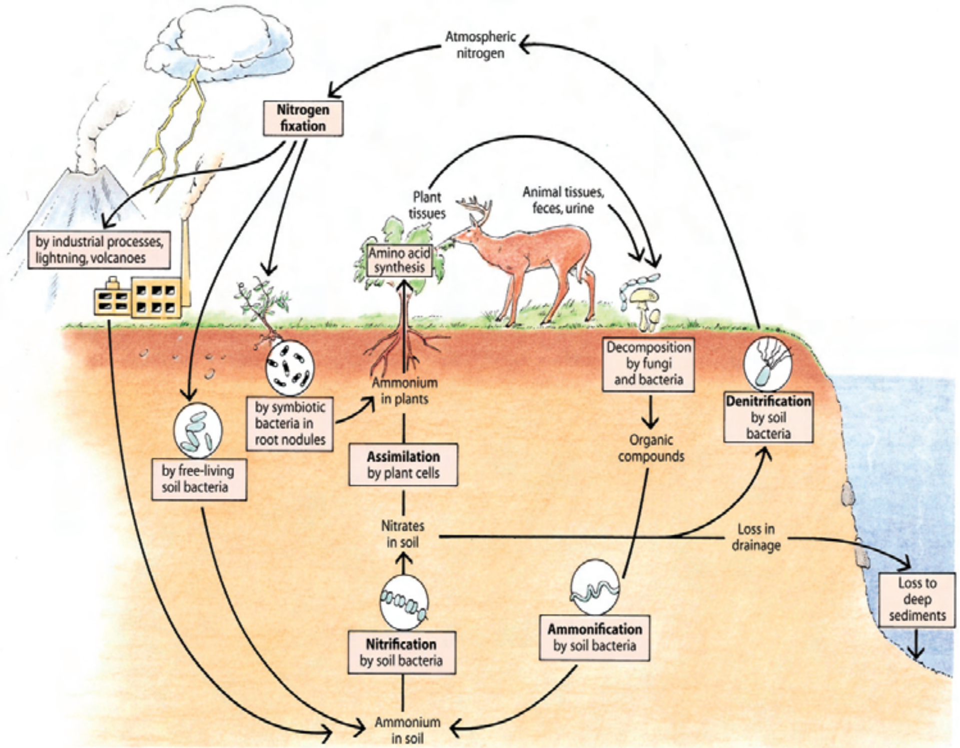


Figure 4-2. The Nitrogen Cycle. [From **122**].

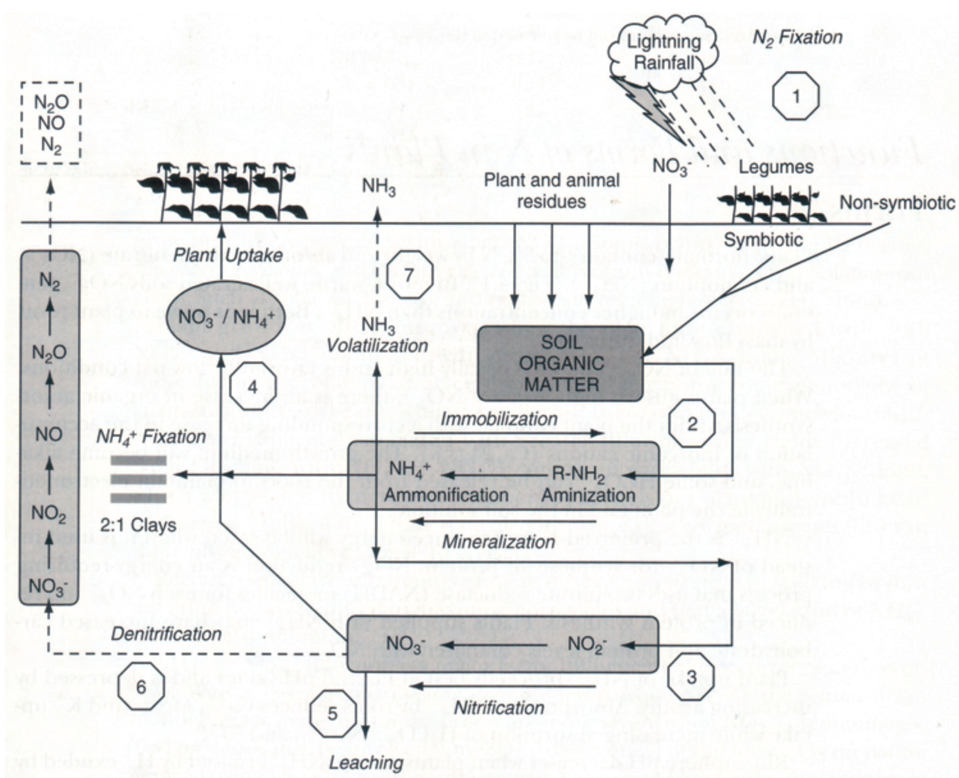


Figure 4-3. The Nitrogen Cycle. [From **73**].

downwind. The magnitude of the nitrogenous depositions across the state is highly variable, ranging from 1-45kg/ha/yr [**30**]. For comparative purposes see **Table 4-3**.

Table 4-3. Relative Magnitude of Nitrogen Deposition

Location	Year	Kg N/ha/yr	Reference
Santa Ynez Mtns	1978-1979	**1-2**	**131**
San Bernardino Mtns	1992-1996	**5-31**	**57**
San Gabriel Mtns	1980-1994	**12-23**	**57**
So. Sierra Nevada Mtns	1992-1999	**6-17**	**57**
Lake Tahoe Basin	1999	**4-5**	**57**

Emissions, initially atmospheric inputs, are deposited on surfaces during summer, and infiltrate through the soil profile during winter rainfall events. Some compounds increase soil N levels and are available for plant uptake [**116**]. Other N compounds are not in forms useful to plants and some may be harmful.

Some absorption of N pollutants is thought to occur through foliar means in addition to the more typical root pathway [**71**,**113**,**164**].

Watersheds at risk of becoming N-saturated —the point at which the N retention capacity by biota and soil chemical fixation mechanisms are exceeded [**142**]— may export above normal amounts of N, resulting in water quality deterioration in both ground water and streams [**56**]. Chronic N deposition has the potential to alter ecosystem processes including growth stimulation, induced nutrient deficiencies, direct phytotoxic effects, shifts in root/shoot ratios, effects on soil biota and altered drought stress and frost tolerance [**29**]. Ultimately continued N deposition affects the health and sustainability of ecosystems, including changes to species composition [**30**].

4.1.5.2 Biological Nitrogen Fixation.

Biological processes account for some 60% of global N_2-fixation [**176**]. A limited number of prokaryotes, some free-living (nonsymbionts), some rhizospheric symbionts, can take advantage of the abundant reservoir of atmospheric nitrogen because they can assimilate N_2 directly. Soil N is replenished primarily by this process (**Table 4-4**).

Table 4-4. Estimates of Relative N Inputs from Atmospheric Deposition & Biological Nitrogen Fixation (BNF).

Expressed as kg N/ha/yr. [From **17**]

Atmospheric Deposition Bulk Precipitation	BNF: nonsymbiotic	BNF: symbiotic
1-12	< 1-5	~ 10-160
not influenced by industrial emissions		nonagricultural ecosystems in early successional stages

4.1.6 Legume-Symbiont N_2 Fixation

Leguminous plants (legumes), among many groups of plants, have the potential to increase the levels of bioavailable N in the soil via a symbiotic association with particular bacteria that can convert atmospheric nitrogen into a biologically useful form. Legumes can form symbiotic, mutually beneficial, partnerships with rhizobia. These rhizobia can recognize specific plants, penetrate plant roots (occasionally stems), provoke development of a root nodule, transfer into a host cells and then proceed to convert atmospheric nitrogen (N_2) directly from the gaseous component of the soil matrix. Atmospheric nitrogen (N_2), otherwise unavailable to plants, is thus converted into a forms —ammonium ions (NH_4^+ and NO_3^-)— readily usable by the plant.

The accumulated N is available not only to the host legume, but also can be transferred to any other plants in the immediate vicinity for a short time via the rhizosphere [**91**] through direct root excretion as amino acids [**41**], microbial decomposition of legume material [**95**] or direct transfer via mycorrhizal associations [**41**]. On a land area basis N-transfer amounts have ranged from 29 to 53kg N/ha/yr in perennial legume-grass mixtures in Minnesota [**75**]. Even though the N-transfer amounts can be significant [**51**], transfer is spatially localized, confined to neighbors in close proximity [**4**]; thus, nitrogen gains are spatially patchy and not equally distributed across the landscape.

A tremendous potential for contribution of fixed N_2 to soil ecosystems exists among legumes [**117**]. Legumes are very important both ecologically and agriculturally because they are responsible for a substantial part of the global flux of N from atmospheric N_2 to fixed forms. Successful native legume-rhizobial symbioses definitely increase the incorporation of fixed N_2 into soil ecosystems [**176**]. In areas dominated by symbiotic nitrogen fixers, whether agricultural or wildland, there can be very high rates of N_2-fixation, often exceeding 100kg N/ha/year [**163**].

In addition to the potential for converting significant amounts of N_2 to bioavailable forms, the process can begin soon after legume germination. Nodule formation can occur in 5-6 days after exposure of actively growing legume roots to the appropriate bacteria and then N_2-fixation may initiate as soon as 8-15 days thereafter [**66**].

This symbiosis between legumes and bacteria is limited by varying degrees of host preference or specificity. If legumes are seeded into a substrate that lacks the appropriate bacteria, N_2-fixation may not be achieved [**100**]. To increase the likelihood of nodule formation and maximize N_2-fixation, the practice of inoculation, which coats legume seeds with a commercial preparation of effective bacteria at the time of planting, has been widely employed throughout agricultural and revegetation systems [**33**]. Thus, there are two main conditions where inoculation is useful: 1) when there are no indigenous strains of the required rhizobia in the soil, and 2) when the level of the required rhizobia in the soil is low [**33**].

In theory, effective seed inoculation need be practiced with only the first year's seed application. Nodules age and senesce some 50-60 days after formation; if the legume is still actively growing, new nodules may be formed. When nodules senesce and release bacteria into the rhizosphere, these bacteria have the potential to persist in the soil and serve as future inoculants for the next cohort of legumes. Persistence, however, is variable and the percentage of nodules formed by commercial inoculant strains declines over time due to competition with indigenous rhizobia [**52**,**63**,**84**,**102**,**107**,**128**].

4.1.6.1 Legume Inoculation In Agricultural Systems

The practice of legume inoculation has been carried out primarily to provide a cost-effective bioavailable N source for such agricultural purposes such as crop production or managed pasture grazing, in order to maximize plant yield, where, owing to harvest, there is a continual requirement for N replenishment. Because N is the major limiting nutrient in most agricultural systems, inoculated legumes, which can decrease the need for costly N fertilizer applications, are an integral component in both forage mixtures and crop rotations. Under an agricultural cropping regime plants are grown in a monoculture of exclusively one species at a time and at very high density (>100,000 plants/ha). These plants are then harvested unless grown for green manure. In either case the rhizobia that have nodulated are released back into the soil and are available to nodulate again [**66**]. In intensely managed pasture systems legumes and grasses may be grown simultaneously in very high densities with both receiving inputs of fixed N_2 [**135**].

4.1.6.2 Legume Inoculation In Non-Agricultural Systems

Results of studies in non-agricultural ecosystems [**36**,**83**,**92**,**146**,**150**] indicate that N_2-fixing symbionts may:

- Contribute to the long-term accretion of soil N and organic matter on infertile sites;
- Enhance nutrition of associated plant species when N is limiting;
- Stimulate primary production and biomass accretion;
- Alter a variety of biogeochemical processes.

These effects are further influenced by successional processes, soil N availability and soil organic matter [**17**]. Increases in N availability associated with N_2 symbiont fixation may also alter a number of soil ecosystem processes:

- Immobilization rates of N, P, S [**114**];
- Allocation rates of aboveground biomass [**10**,**11**];
- Increased litterfall mass [**18**];
- Increased above ground nutrient pools [**17**];
- Reduced C:N ratios [**171**];
- Increased concentrations of inorganic N [**17**];
- Accelerated leaching of organic and inorganic N [**108**].

Note

Legumes are not obligately dependent on the presence of the rhizobial association for survival as may be otherwise incorrectly inferred from the preface of Bulletin 1842 [**118**].

If nutrient levels, especially N, in the soil are adequate for plant growth, the legumes will establish and function perfectly well without a rhizobial symbiont. Inorganic N will be obtained from the soil matrix and not from the process of N_2-fixation. However, in such circumstances, there will be no supplementation of the available soil N levels by the N_2-fixation process via the legume-symbiont association.

4.1.6.2.1 Surface Mining Systems. Surface mining completely destroys ecosystems [**173**]. Re-establishment of vegetation on surface mined lands depends upon the development of soil and the soil processes that affect water and nutrient availability. Reduction in organic matter along with erosion have profound negative impacts on the diversity and abundance of those soil biota which form the basis of the decomposition and mineralization processes in arid ecosystems. In reclamation of severely disturbed soils N_2-fixing species such as legumes have been widely used in the initial stages of revegetation and as a long-term treatment [**19**,**20**].

4.1.6.2.2 Rangeland Systems. The harvest of livestock from open rangeland grazing systems has also been recognized as resulting in nutrient removal from ecosystems [**153**]. Rangeland improvement via grass seeding efforts have often included inoculated legume mixtures to provide additional inputs of N for the livestock and to counteract potential soil nutrient depletion [**61**] and disruptions in the trophic relationships among the soil biota. Under northern California rangeland conditions, effectively nodulated legumes (clovers) can potentially fix significant quantities of nitrogen, equivalent to the addition of 250lbs of ammonium sulfate/acre [**118**].

4.1.6.2.3 California Highway Revegetation Systems. On post-construction roadsides where revegetation is attempted from seed applications that often include inoculated legumes, Caltrans holds three broad, implied goals and expectations:

1. Rapid Vegetation Cover Establishment (70% cover during first rain season);
2. Vegetation Persistence;
3. Succession to Long-Term Context Vegetation.

4.1.6.2.3.1 Rapid Cover. For Caltrans, revegetation requires overcoming a host of conditions hostile to plant growth including, but not limited to, depleted levels of nitrogen. To accomplish an initial plant cover of roadsides Caltrans often specifies the use of inoculated nitrogen fixing legumes as part of a hydroseed mix consisting of an assemblage of plant species possessing attributes that foster rapid coverage: high germination rate and fast growth with ample foliar coverage. The majority of these species are non-native plants with winter annual life cycles, that is, they germinate in response to late fall or winter rains, grow throughout the winter and early spring, setting seed and senescing shortly after the rains have ceased. The cycle is repeated every year as long as nutrients and rain remain adequate.

The main problem with annual plants is the lag time between the initial onset of rains that trigger germination and achieving enough vegetative growth to effect cover. In the absence of cover soil loss and N leaching occur.

In addition fertilizers, including N, are usually specified for the initial hydroseed mix. Depending on the levels of N in the roadside soils, the legumes may or may not participate in N_2-fixation; high levels of N would suppress N_2-fixation. Fertilizer quantity is estimated to be sufficient for 1-2 years of plant growth and has the built-in assumption that nitrogen inputs from legumes and nitrogen cycling will be adequate to maintain plant growth beyond the fertilizer time limits.

4.1.6.2.3.2 Vegetation Persistence. Vegetation persistence under an annual plant cycle requires a continually renewed seed bank and appropriate annual germination and growth conditions. Interruptions at any of these stages will ultimately reduce vegetation cover. The maintenance of plant cover over time requires resumption of the key soil processes of decomposition and mineralization as mediated by soil microbiota [**173**] requiring some minimal levels of soil organic matter to sustain the microbiota. The alternative is the annual input of supplemental nutrients via industrial fertilizers.

Legume persistence is influenced by environmental conditions [**95**]. Some of the annual legumes in the Caltrans seed mixes (*Lupinus* spp., *Lotus* spp.) germinate best under high light, post-disturbance conditions. Establishment is significantly higher in newly disturbed or nonvegetated conditions as opposed to even a low density of established native grasses [**27**] or in second and third year post-fire revegetation [**68**]. Furthermore, even if legumes do continue to germinate in subsequent years, the rate of legume-rhizobia N_2-fixation decrease with shading from other vegetation. Thus, any N gains from N_2-fixation by annual legumes would likely be greatest during the first year of revegetation unless other vegetation cover was exceptionally sparse. Using perennial legumes such as Deerweed (*Lotus scoparius*), Yellow Bush Lupine (*Lupinus arboreus*), or Spurred Lupine (*L. caudatus*) could extend the N inputs somewhat as long as shading by other plants was minimal. Overall the legume-symbiont N gain is presumed to be most effective in the first year of growth, decreasing with time as other vegetation becomes dominant.

4.1.6.2.3.3 Succession. Conventional wisdom concerning the role of N_2 fixers as facilitating primary succession (such as from barren post-construction roadsides) has been based on studies documenting the increase in nitrogen availability within stands of N_2-fixing pioneers [**157**,**162**]. Other studies have found that although nitrogen availability may be increased, succession is not always facilitated and is sometimes even inhibited [**109**].

The general expectation that rapid cover and subsequent persistence of non-native annual plants will provide the environmental conditions that lead to native shrub establishment, i.e., a facilitation model of succession, is often not the case. Succession does not necessarily follow a trajectory towards a pre-disturbance state [**2**,**40**,**82**,**144**]. The assumption that once the physical environment is re-established, natural succession processes will return the biotic system to its original condition is no longer valid. The dynamics of a degraded ecological condition that affect all the elements influencing restoration appear very different from those of an undisturbed ecological condition [**145**].

Such disturbance dynamics likely influence Caltrans roadside revegetation. Once annual non-native grasses are well-established in the semi-arid mediterranean-type climate of California their populations are self-sustaining [**42**,**168**]. Nitrogen-rich soils are conducive to the establishment and maintenance of exotic annual species rather than native perennials [**35**,**46**,**72**,**85**]. While some native shrubs do recolonize annual grasslands under some conditions [**59**], other annual grasslands show no evidence of shrub recolonization after 10 [**179**] or even 70 years [**180**] post-disturbance. The ability of shrubs to establish among annual grasses may vary by site and species [**54**] and at least in some situations the presence of annual grasses has reduced [**54**] or precluded (**178**) shrub recruitment. Thus, the persistence of annual species and the lack of shrub recruitment within a typical successional time (<25 years) is to be expected [**70**,**90**]. While successful recruitment of shrubs has and will continue to occur within some roadside conditions, it should never be considered as the inevitable outcome of a single hydroseed mix application that has rapid cover as the primary goal.

4.1.6.3 Need For Inoculation

The decision to inoculate legume seeds should be based on a demonstrated need from experimental plots or as insurance against crop or pasture failure [**47**]. Field assays [**7**,**21**,**24**,**44**,**147**], however, can require from one to many months to complete. The question of the need to inoculate is multifaceted and has been approached in the agricultural context from three perspectives: historical [**3**], microbiological [**126**], and soil [**148**]. These are summarized in **Table 4-5**, with selective modification to reflect the Caltrans roadside revegetation perspective.

4.1.6.4 Inoculation Methods

Rhizobia may be introduced to legumes by inoculating the seed or soil. Seed may be inoculated immediately prior to sowing or custom inoculated by local seed vendors with coating facilities to be sown within a week [**47**]. Alternatively seed may be commercially inoculated and stored prior to its sale (pre-inoculated).

Table 4-5. Diagnosis of the Need to Inoculate Roadside Revegetation.
[Adapted from **24**].

Indicators	Assessment Method
Historical	
1. Site History: presence of the same or symbiotically related legumes	Botanical survey
2. Previous revegetation failures owing to low soil fertility	Site assessment Soil fertility assay
3. Land reclamation where topographic reconfiguration has buried topsoil; revegetation expected to occur on subsoil or parent material	Review engineering specs Post-construction soil analysis
Microbiological	
1. Specificity of legumes in seed mix in their rhizobial requirements	Compile database from published literature, manufacturer data
2. Likelihood of effective rhizobial presence	Botanical survey
Soil	
1. Size of the resident population of competitive rhizobia	Microbial field assay
2. Level of soil nitrogen (nitrate)	Soil fertility assay

Box 4.1 summarizes legume inoculation techniques. These procedures are used in association with crop plants, and have been applied with variable success to the inoculation of legumes grown in natural or revegetation settings. For the purpose of roadside seed application and soil stabilization on sloping terrain, hydroseeding is the most frequently used method by Caltrans, rendering the techniques of Soil Inoculation unsuitable. *UC-AES Bulletin 1842* recommends pelleting as the preferred technique for rangelands.

The equipment and processes used by contractors or seed vendors may vary but the goals of the seed coating process are to produce consistently evenly coated seeds. Research into alternative seed inoculation formulations is on-going but most alternatives have not been adopted by the inoculant industry because of the high cost of technical handling [**6**].

Box 4.1
INOCULATION TECHNIQUES

SEED INOCULATION
Inoculant mixed with milk or mild adhesive material, dried in the shade and sown the same day; requires the inoculant strain to be packaged in a relatively fine carrier material or liquid that will adhere to the seed.

Dusting

Peat inoculant mixed with seed without re-wetting.

Most inoculant dislodged by sowing machinery.

Slurry

Seed mixed with water solution of peat, often with an adhesive.

Retains more inoculum on seed coat.

Lime or Phosphate Pelleting

Seed treated with slurry peat inoculant & adhesive, then coated with calcium carbonate (superfine limestone) or rock phosphate.

Improves rhizobial survival in delays up to one week between inoculation and sowing; counters acidic effects of soil or fertilizer; can be used for aerial sowing.

Some studies indicate that gum arabic and methyl cellulose alone were better than limestone (calcium carbonate) due to the high pH of limestone.
Should use limestone with a pH as neutral as possible.

Vacuum Impregnation

Rhizobia introduced into or beneath seed coat under vacuum conditions.

SOIL INOCULATION
Direct inoculation of seedbed, usually rows or furrows, at time of sowing with granular peat or liquid.

- avoids damage to fragile seed coats
- overcomes adverse effects of biocides applied directly to seed
- reduces losses of inocula caused by seed drilling equipment
- allows highest inoculation rates
- improved survival when there are delays between sowing and germination.

Liquid Inoculation

Peat culture mixed with water or liquid inoculant applied to the seedbed (liquid inoculants may also be applied to seed).

Granular Inoculation

Granules containing inoculum sown with seed in seedbed

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

1. Inoculated legume seeds can be obtained by one of three methods:

(A) by purchasing pre-inoculated seeds
(B) by contracting for custom inoculation services
(C) by the rancher pelleting seeds on-site.

Method A is not recommended because the shelf-life of rhizobia on inoculated seeds is very short.

Method B can be successful, but the supplier and the expenses must be investigated carefully.

This bulletin is directed toward helping individuals use Method C to pellet their own legume seeds.

4.2 Inoculation Provider

Caltrans recognizes three options for inoculation execution:

- **pre-inoculation** by commercial/industrial professionals;
- **custom inoculation** by the seed vendor or contractor;
- **ad hoc** inoculation by the revegetation landscape contractor.

Each option can be effective, but the set of potential problems associated with each factor in the inoculation process may not be equivalent among the options, e.g., a professional laboratory setting may have more precision equipment for effecting inoculation than the landscape contractor. Minimization of any of these problems is dependent upon the equipment and abilities of each individual provider.

Table 4-6 lists the potential problems by category, *references* the subsection which details the potential problems for that category, *indicates* the degree to which the Standard Specification 2002-2.10 currently addresses the problem, and *provides* a very generalized estimate of which provider option may offer the most effective problem mitigation, although this is prone to being highly variable.

Table 4-6. Synopsis of Potential Problems With Inoculation Factors Relative to Provider Category.

Problem Potential Among Providers : = nearly equivalent ▲ increases problem ▽ decreases problem

Sub-section	4.4	4.5	4.5	4.7	4.7	4.3	4.6	4.6	4.6
Factor	Inoculant Purity	Shelf Life	Storage Conditions	Post-Inoculation Seed Storage Time	Post-Inoculation Seed Storage Conditions	Strain Selection	Rate of Inoculation	Inoculation Equipment & Expertise	Inoculation Check
Factor Affects	Viability					Effectiveness			
Provider									
Pre-Inoculation	=	=	▽	▲	▽	=	▽	▽	▽
Custom Inoculation	=	=	=	=	▲	=	▽	▽	▲
Landscape Contractor	=	=	=	=	▲	=	▲	▲	▲
CT SPEC Control	No	Yes	No	Yes	No	Partial	Partial	Partial	No

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

2. Select a good commercial peat inoculant that contains root-nodule bacteria specific for the legume to be planted. Make sure the legume species is named on the label. No other inoculant is suitable.

4.3 Selection of Rhizobial Strains

Rhizobial bacteria exhibit varying degrees of specificity and fidelity to individual legume host plant. Thus, correct association of rhizobia to legume species used is a minimum requirement for proper inoculation.

4.3.1 Host Specificity

Symbiont partner preference, i.e., host selectivity, between legumes and rhizobia has been long appreciated and formed the basis of the traditional "cross-inoculation" classification systems early as 1896 [**47**], whereby some 7, and later up to 20, groups of legumes with agricultural significance, were matched with effective rhizobial strains. The specific epithet of the microsymbiont reflected in most cases the corresponding host plant group, even though some anomalies had been known. More recent work has shown the situation to be far more complex than previously understood. Degree of host specificity varies markedly among rhizobia [**155**]; one strain of rhizobium may be effective with only one species of legume, while another strain may be effective across multiple genera.

4.3.2 Nodulation

Even nodulation and N_2-fixing ability are not 100% correlated [**78**]; the ability to nodulate does not guarantee that N_2-fixation will occur [**65**]. Currently more than 100 different strains of rhizobia are needed to satisfy the inoculation requirements of important agricultural legume species [**66**]. While inoculation is routine for agricultural legumes, the inoculation of natural ecosystem legumes with an appropriate strain(s) of rhizobia presents problems not encountered in agricultural situations. Selection of an appropriate rhizobium is critical [**140**] and yet research on rhizobia which nodulate non-agricultural legumes of significance only to the nitrogen cycle has been limited [**66**,**87**,**93**,**94**]. Graham et al. [**66**] reported that the conventional cross-inoculation groupings were not effective with indigenous shortgrass prairie legumes and that transfer of rhizobia could occur among hosts in the prairie environment.

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

14. Dig a few seedlings after the legumes have produced three or four leaves. The type and pattern of nodulation can give useful information:

- A few large nodules on the crown or upper root indicate early, effective nodulation, provided the nodules are pink inside.
- Lack of nodulation usually indicates some fault in the inoculation or sowing technique. Review the above instructions.
- Many small, white nodules scattered over the entire root system also suggest an inoculation problem, and indicate the presence of ineffective root-nodule bacteria in the soil. A good peat inoculant and proper inoculation technique should eliminate this problem.

The effects of native legumes on soil fertility through effective nodulation are highly variable [**41**,**88**,**175**,**177**]. In contrast to "best case" agricultural N gain of often exceeding 100kg N/ha/year [**163**], Deerweed (*Lotus scoparius*), a common N_2-fixing suffrutescent perennial in cismontane California that is specified in seed mixes by Caltrans, has the potential to fix only 10-15kg N/ha/yr [**112**]. The reduced quantity reflects in part the temporal limitations of a brief spring period in California ecosystems where the coincidence of soil moisture and temperature are favorable for plant growth, as opposed to an irrigated or summer rain agricultural context. The brief duration of the growing season will limit the N_2-fixation

Note

UC-AES Bulletin 1842 (see **Box 2.4**, number 14) recommends evaluating inoculation success by extracting legume seedlings to look for pinkish nodules on developing roots. This method can be inadequate because the same rhizobia species can interact with the same host as:

- a **mutualist** (N_2-fixing for the host),
- a **non-symbiont** unable to infect and nodulate with the host, *or*
- a **parasite** that infects the host but does fix significant amounts of N_2.

Ninety percent of all inoculant has been found to have no practical effect on the productivity of the legumes for which it is used.

Figure 4-4. Well-Nodulated Root System.

[From **122**]

potential of both drought deciduous perennial legumes such as deerweed, and annual legumes such as the lupines commonly used in Caltrans seed mixes.

Unfortunately using inoculated legume seed does not guarantee formation of effective nodules or N fertility improvements to the soil. Nitrogen fixation can be limited by numerous environmental factors that affect either partner. Effective nodulation is a function of the number of rhizobia applied to the seed and their subsequent survival both on the seed and in the soil before seed germination [**129**]. **Figure 4-4** shows a well-nodulated root system. The process of nodulation is vulnerable to deleterious conditions in the inoculation and storage phase, seed application phase and on-site post-application phase. Ninety percent of all inoculant has been found to have no practical effect on the productivity of the legumes for which it is used [**24**].

Successful high rates of N_2-fixation are best documented from agricultural situations where most research has been concentrated. While there is a wealth of information on the molecular biology of N_2-fixation and its regulation for the few well-studied crop legumes, there is but scant information on ecological controls of symbiotic N_2-fixation in natural ecosystems [**17**,**163**]. In addition, information on wildland rhizobia is minimal [**66**,**104**].

4.3.3 Indigenous Rhizobia

The variability in the effectiveness of rhizobial strains is especially problematic with non-agricultural legumes used for revegetation purposes. While extensive investigations have been effected on cultivated *Trifolium*, *Melilotus* and *Medicago*, owing to their value in pasture and agricultural systems, scant work has been done to characterize the optimal rhizobia for the native California legumes that Caltrans also uses in seed mixes.

Preliminary investigations have identified the indigenous rhizobia for: **1**) three desert woody legumes, Whitethorn, *Acacia constricta*, Mesquite, *Prosopis glandulosa*, and Desert Ironwood, *Psorothamnus spinosus* [**166**]; **2**) *Lotus purshianus* and *Lupinus bicolor* from central Sierra Nevada foothills [**182**]; **3**) four native clovers, *Trifolium ciliolatum, T. microcephalum, T. tridentatum*, and *T. variegatum,* from central Sierra Nevada foothills [**183**]; and **4**) four native clovers, *Trifolium bolanderi, T. longipes, T. monanthum*, and *T. wormskioldii,* from central Sierra Nevada meadows [**169**,**181**]. These studies investigated rhizobial population diversity and host specificity, but not efficiency of N_2-fixation.

Plant response to inoculation is affected by the presence and quality of indigenous rhizobial populations [**16**,**50**,**69**,**136**], physiochemical constraints [**79**,**137**] and climatic conditions [**32**]. Thus, rhizobial strain selection criteria must include 1) competitiveness with indigenous rhizobial populations, and 2) "ecosystem response" factors pertinent to specific geographic, soil and environmental regions [140]. Less than one strain in 100 is likely to meet the standards, so rhizobia must normally undergo extensive laboratory, greenhouse, and field testing [65]. No literature was found for such characterization *in situ* for native California legumes used in revegetation.

Problem 4.3.1 ► Lack of Standards

- **Lack of specificity about degree of compatibility or effectiveness of N_2-fixation**
- **Lack of industry-wide standards regarding compatibility or effectiveness**
- **Strains vary among manufacturers**

Rhizobial strains compatible with particular legumes may successfully form partnerships, but the rhizobium may not necessarily be efficient at N_2-fixation under roadside conditions. It is well-documented that effectiveness of N_2-fixation is highly variable among strains that will cause nodulation and this is especially true under field conditions where there may be indigenous rhizobia already present. Strains vary among manufacturers and there are no National, State or industry-wide compliance standards to which any product can be held accountable.

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

Pre-test legumes for each project to demonstrate and quantify the effectiveness of the strains being used under particular roadside conditions.

Problem 4.3.2 ► Lack of Guidance

- ***UC-AES Bulletin 1842* lacks guidance regarding rhizobial strains for the native legumes often called for in Caltrans revegetation seed mixes.**

  Most research regarding symbiotic N_2-fixation has been carried out within an agricultural context. Hence, there is scant information on wildland rhizobia and the ecological controls of symbiotic N_2-fixation in natural ecosystems. Rhizobia that are compatible and effective with agricultural legumes may not be effective with California native legumes.

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

Provide for field recovery and identification of nodule occupants of native legumes. As *UC-AES Bulletin 1842* recommends, strains of rhizobia to be used as inoculants can be isolated from nodules on vigorous legumes. These strains should be selected from the general geographic region in which the inoculants are to be used, then tested under controlled conditions.

BULLETIN 1842

SEED INOCULATION AND FIELD PROBLEMS

The *UC-AES Bulletin 1842* recommends contacting the local UC Extension Farm Advisor Office for manufacturer information on inoculant purity.

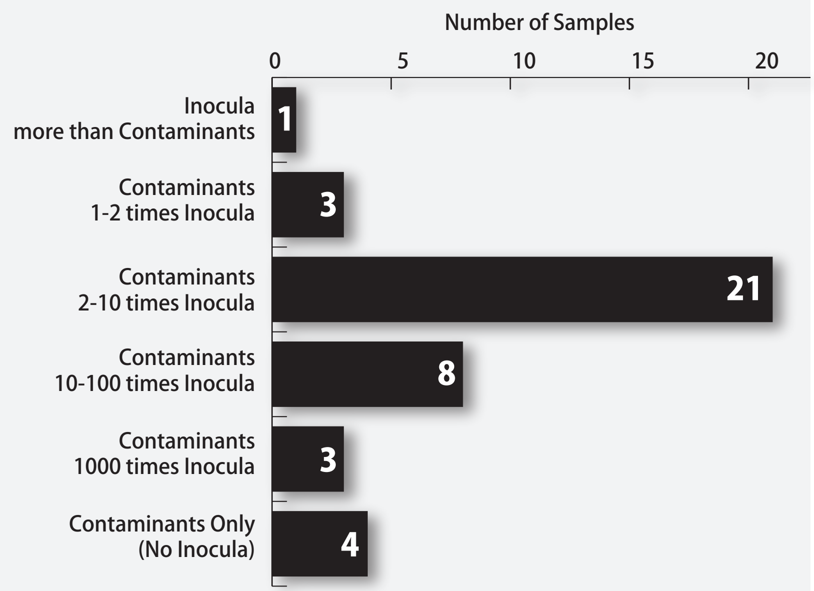
4.4 Inoculant Purity

Inoculant cultures manufactured in North America are noted to suffer from both poor numerical quality and questionable purity. Reportedly there are substantial differences among commercial inoculants and quality control methods. Many have been shown to be unsatisfactory due to contamination.

Many factors contribute to high quality legume inoculant products, with the most important being high numbers of live rhizobia capable of nodulation and N_2-fixation with the target host, and minimal or no contamination [**99**]. Independent evaluations suggest that most cultured inoculant manufactured in North America is of relatively poor numerical quality and of questionable purity [**33**,**115**,**130**,**138**,**159**]. Significant differences have been found among various commercial inoculants and quality control methods [**129**]. A survey of North American industrially-manufactured inoculants found a substantial percentage of inoculants were unsatisfactory due to high levels of contamination with other bacteria, actinomycetes and fungi. Levels of rhizobial purity varied widely, with only one of forty (1/40) samples containing more rhizobia than contaminants and 4/40 samples with too few rhizobia as to be detectable (**Figure 4-5**). Contaminants within the inoculum not only reduce the numbers of viable rhizobia available to coat seeds, but can also decrease the survival of any rhizobia present and shorten shelf life [**45**].

Figure 4-5. Rhizobial Inoculant Purity.

[Adapted from **115**]



Box 4.2

TYPES OF INDUSTRY-WIDE INOCULUM QUALITY CONTROL REGULATION

Legislation & Enforcement

Country	Reference
Brazil	149
Canada	115
France	165
Uruguay	22

Voluntary Manufacturer Participation

Country	Reference
Australia	99
India	22
Netherlands	139,140
New Zealand	139
Russia	139,140
South Africa	99,101
Thailand	139,140

Manufacturer Discretion

Country	Reference
United Kingdom	47,99
United States	22,47,99

Unfortunately the widespread availability of legume inoculants has not resulted in international standards for quality control nor rates for use [**140**].

Large degrees of variation in standards exist throughout the global industry [**24**,**47**,**99**], ranging from countries with regulatory legislation and enforcement, to voluntary manufacturer participation with industry-set standards, to being left to the manufacturer's discretion (**Box 4.2**).

Problem 4.4.1 ► Lack of Purity Standards

- **Lack of industry-wide standards regarding purity**
- **Purity varies among manufacturers**

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

- Require a purity test to accompany every inoculant purchase.
- Work with manufacturers to improve and ensure purity standards.

The *UC-AES Bulletin 1842* recommends contacting the local UC Extension Farm Advisor Office for manufacturer information.

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

4. Check the freshness of the inoculant by referring to the expiration date printed on the container. The inoculant is a living culture of root nodule bacteria that can be killed by drying and by high temperatures. Make sure the culture has been stored under refrigeration. Poor storage conditions can cause nodulation failure by reducing the number of viable bacteria in the inoculant.

4.5 Inoculant Shelf Life and Storage Conditions

Viability is monitored solely by the expiration date (shelf life) as determined by the manufacturer. Commercial cultured rhizobial inoculants are living preparations with limited shelf lives. The number of living rhizobia per package decline quickly over time [**33**]. Contaminants within the inoculum also decrease the survival of rhizobia and shorten the shelf life [**45**]. To slow the rate of loss, the quality of the storage conditions must be closely monitored; usually this requires refrigeration at an optimum temperature as indicated on the package by the manufacturer.

Problem 4.5.1 ► Expiration Date

Standard Spec 2002-20-2.10 requires only the date of inoculation to be shown on the package, however *UC-AES Bulletin 1842* states that the expiration date on the inoculant container should be observed.

Options

SPECIFICATION REVISION

- Revise the Standard Specifications to require monitoring both the expiration date provided on the package as well as the date of inoculation.
- Should the expiration date occur before the inoculation date, the inoculated seeds should be rejected by the Project Engineer.
- Should the expiration date occur before the scheduled project seed application date, the inoculated seeds should be rejected by the Project Engineer.

The *UC-AES Bulletin 1842* recommends contacting the local UC Extension Farm Advisor Office for manufacturer information.

Problem 4.5.2 ► Inoculant Storage

There are no requirements to ensure the inoculant has been stored appropriately so that the bacteria meet a quantitative minimum standard of viability.

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

Options

Require a quantitative test of minimum viability standards performed by a laboratory immediately prior to the Project Engineer accepting the product from the landscape contractor.

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

4. Use the inoculant at the rate the manufacturer recommends on the package, or, even better, at four times the recommended rate. Never use a lower amount, or there will be too few bacteria on each seed to produce good nodulation.

UC-AES Bulletin 1842 **provides no guidance for inoculation of native legumes.**

4.6 Rate of Inoculation

The rate of inoculation, i.e., the minimum number of rhizobial inoculants per seed, is critical to improve the probability of nodulation. In general, when the number of viable rhizobia inoculated per seed increases, nodulation is improved [**33**]. Exact numbers required are not definable, but in this case, the axiom "more is better" is true. Estimates of optimum numbers of rhizobia per seed are extremely variable, attaining up to 1 million for large-sized legume seeds.

An analysis of drill-planted lupines showed an increase in the percent of nodulated plants when the log number of inoculant was increased from 4.4 to 5.3 per seed [**119**]. The most widely accepted inoculant standards for numbers of rhizobia delivered per seed area are 10^3 (1000), 10^4 (10,000), and 10^5 (100,000) rhizobia for small, medium, and large sized seeds, respectively. Other list minimum goals as 10,000 rhizobia/seed for clover-sized seed to 1 million rhizobia per seed for soybean-sized seed [**65**]. Evaluations of inoculant products to achieve these numbers are not currently standardized [**99**]. Small-sized seeds can accommodate fewer numbers of rhizobia than large-sized seeds.

UC-AES Bulletin 1842 cites 1,000 rhizobia per seed as a minimum and provides a comparison of inoculation methods with results ranging from 925-656,000 bacteria per seed. To achieve this it recommends using 4 times the suggested application rate listed on the inoculant package. Caltrans requires legume seeds to be pelleted at a rate of 2kg (2lbs) inoculum/100kg (100lbs) legume seed. Individual project specifications [raw data from **34**] confirm project specification instructions of this minimum and in some situations the minimum rate has been increased from 2 to 5 times the suggested application rate.

Problem 4.6.1 ► No Effectiveness Monitoring

- **Independent evaluations of inoculant products have shown that with a lack of industry-wide standards it is likely that suboptimal rates of inoculation can occur even with appropriate application rates.**
- **There is no monitoring to quantify the effectiveness of the inoculation, i.e., the average number of rhizobia that adhered to seeds for any given project.**
- **Thus, there is no mechanism by which a contractor or seed vendor to know if their inoculation methods have been successful or whether the product met target levels.**

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

Require testing of seed inoculation product by a contracted laboratory to evaluate the average number of rhizobia per seed for each legume species inoculated.

The *UC-AES Bulletin 1842* under sidebar "Pellet Inoculation of Legume Seed" provides quantification for differential amounts of seed, adhesive, and calcium carbonate to be used with the commonly cultivated rangeland legumes, clovers, vetch and alfalfa, using seed size as the discriminating factor (**Box 2.2**). There may be additional quantitative instructions on inoculant packaging.

Problem 4.6.2 ► Native Legumes

- ***UC-AES Bulletin 1842* provides no guidance regarding seed size or mixture quantities for the California native legumes often specified in roadside seed mixes.**
- **Inoculant packaging with quantitative instructions may be too general for species complexes with substantial variation in seed size; agricultural species may differ in seed size from native wildland species resulting in an inadequate rate of inoculation.**

Options

SPECIFICATION REVISION

To standardize methods and improve inoculation rates Caltrans could supplement the *UC-AES Bulletin 1842* list and provide the quantitative data for all species of legumes called for in a seed mix, including native legumes.

4.7 Post-Inoculation Seed Storage

Post-inoculation seed storage duration and conditions are closely linked to inoculum viability. Death of all species of rhizobia on inoculated seed occurs rapidly, particularly when environmental conditions are unfavorable [**25**,**47**]. High mortality occurs due to heat and dessication. At 38˚C, 99.9% of inoculum on seed died between inoculation and sowing the seed [**25**]. In another trial only 5% survived the 3.75 hour delay between inoculation and sowing [**129**]. *Bradyrhizobium* sp. (*Lupinus*) showed a decrease of one log unit in the number of viable rhizobia after only one hour on the seed and 2 log units after 4 hours [**129**]. Inoculated seeds must be applied as soon as possible and require refrigeration (optimum 5˚C) between inoculation and sowing to maintain rhizobial viability. **Box 4.3** lists the three inoculation options and consequences of the time frame.

Post-inoculation storage duration limitations are stated in the standard specification. Individual project specifications [**34**] have often decreased the maximum storage time from 90 to 30 days.

BULLETIN 1842

SEED INOCULATION AND FIELD PROBLEMS

6. Hold the inoculated seed in a cool, shady place, and plant as soon as possible into a seedbed that will receive a germinating rain in the very near future. If possible, plant into a moist seedbed after the first fall rain. Never plant in the summer months. Always remember that drying kills bacteria in the inoculant and reduces nodulation.

Box 4.3

POST-INOCULATION STORAGE TIME

Vendor Pre-Inoculation [26,121,130]

Commercial Inoculation

Time Frame: duration before sale variable

Potential Problem: poor survival of rhizobia

Custom Inoculation [23]

Inoculation by seed vendor after sale and before delivery of seed

Time Frame: maximum 10 days between inoculation and sowing

Potential Problem: numerical quality of product erratic

Contractor Inoculation

Landscape contractor performs inoculation procedure

Time Frame: must contact Caltrans PE > 2 days before inoculation

Potential Problem: no maximum storage time indicated

Problem 4.7.1 ► Time Limit

The 90-day post-inoculation storage period currently allowed by SS 20-2.10 is far too long. It may accommodate the business practices of industrial/commercial providers that inoculate large batches of seed in advance of specific orders, but it is unnecessary for the custom inoculation method or the landscape contractor inoculation method. It results in a significant reduction in rhizobial viability.

Options

SPECIFICATION REVISION

The Standard Specification should segregate the time limits for post-inoculation storage based upon the inoculation provider. The commercial/industrial provider may require a 90-day window, however, the custom inoculation and the landscape contractor inoculation should be reduced to 7-10 days.

Problem 4.7.2 ► Storage Conditions

Given the potential for complete mortality of rhizobia caused by high temperatures and desiccation during post-inoculation storage, the lack of guidance regarding this stage is unfortunate. *UC-AES Bulletin 1842*, written specifically for a rancher presumably without refrigeration capacity in the field away from the ranch facilities, suggests a shady cool storage location.

Options

SPECIFICATION REVISION

The Standard Specification should better define the post-inoculation storage conditions regarding temperature and humidity in order to keep the rhizobia alive.

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

***UC-AES Bulletin 1842* refers only to pelleted drill-seeding, not hydroseeding.**

4.8 Inoculated Seed Application Method

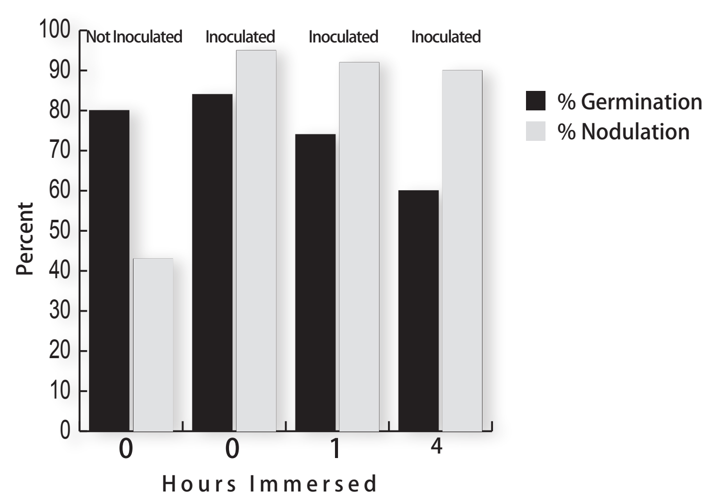
Seed application on slopes is usually done hydraulically. Hydroseeding combines pelleted legume seed, fertilizer, tackifier and wood fiber mulch into a water-based slurry that is then sprayed onto the roadside slopes in a single operation. This method has some potential to damage seed and legume inoculant from:

1) extended immersion in the hydroseeder tank solution,
2) acidity of the solution caused by fertilizers, and
3) physical dislodgement of the inoculant from the seed during agitation and application processes.

When tested to ascertain the effects of long-term immersion, inoculated 'Lana' Vetch seeds showed a decrease in germination of 10% after only 1 hour of immersion and an additional 14% decrease after 4 hours for a total reduction in germination of 24% after 4 hours [**53**]. Of the seeds that germinated, there was a 3% reduction in nodulation with a 1 hour immersion and an additional 2% reduction in nodulation for those immersed for 4 hours for a total of 5% reduction in nodulation (**Table 4-7**). Significant loss of viable rhizobia occur when the hydroseeding slurry has a pH of less than 6 [**28**], whereas rhizobia remained viable when the slurry pH was equal to or greater than 6.

Table 4-7. Effects of Vetch Seed Immersion in Hydroseed Mix.
[Adapted from **53**].
Fertilizer effects: pH shift from 8.5 to 6.5 (near neutral)

Hours in Solution	Inoculated	% Germination	% Nodulation
0	NO	80	43
0	YES	84	95
1	YES	74	92
4	YES	60	90



***UC-AES Bulletin 1842* refers only to pelleted drill-seeding.** This Bulletin was assembled for the purpose of guiding rangeland N-augmentation via inoculated legumes and, as such, is not completely applicable to the Caltrans roadside conditions.

Problem 4.8.1 ► Hydroseeding

Hydroseeding has the potential to damage or dislodge the inoculant from the seed. Some Caltrans project specifications have required the inoculated legume seed to be dry broadcast separately from the hydroseed application [as compiled in 34], but no follow-up monitoring was available to evaluate the effectiveness of that approach.

Options

SPECIFICATION REVISION

Dry broadcasting inoculated legume seed separately from the hydroseed application would likely improve the numbers of viable rhizobia per seed. The sequence of this two-step application would depend upon the germination characteristics of the legumes being used, mostly optimum burial depth, and the thickness and composition of the hydroseed mulch mix.

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

UC-AES Bulletin 1842 **provides no guidance for native legume seeding rates.**

A density of 20 plants per square foot equals how many plants per hectare?

20 plants	*43560 ft^2*	*2.47 acre*	= **2151864 plants**
ft^2	*acre*	*ha*	**ha**

4.9 Inoculated Seed Application Rate

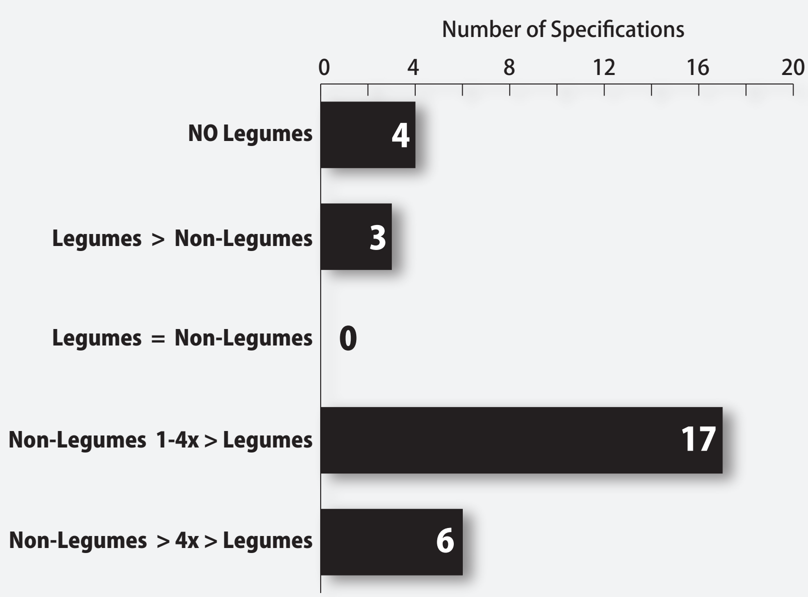
The rate of seeding inoculated legumes on roadside revegetation projects is not indicated by the Standard Specifications, but left to the discretion of the project landscape architect. Rangeland seeding rates recommended by *UC-AES Bulletin 1842* specify a legume density ≥ 20 plants per ft^2 (≥ 871,200 per acre or 2,152,000 per ha).

Agricultural crop monocultures are grown at densities exceeding 1,000,000 plants/ha to achieve 100kg N/ha/yr. The use of a monoculture may not satisfy the roadside revegetation goals of Caltrans which have been multi-species oriented. A high density of legume plantings is necessary to both maximize the potential amount of N_2 fixed and to take advantage of any N-transfer that can occur from legumes to non-legumes in close proximity. Because N-transfer from legumes to non-legumes is highly localized, a high density of legumes is required to effect a significant augmentation to the N pool throughout the roadside area.

Rate of seeding for legumes used in roadside revegetation across the state has been highly variable. **Figure 4-6** shows a graphic summary of legume to non-legume seed rate ratios in 30 selected Caltrans seeding specifications for erosion control spanning the 1990s [**34**]. Caltrans project seeding rates for legumes are highly variable, but typically do not exceed 3 plants per ft^2.

Figure 4-6. Legume to Non-Legume Seed Rate Ratios (kg PLS/ha) in 30 Selected Caltrans Erosion Control Specifications.

[Data compiled from **34**].



Problem 4.9.1 ► Seed Application Rate Guidelines

While the inoculated legume seeding rate would be expected to vary among project sites, there are currently no guidelines for the seeding rates that correspond to levels of N (short- to long-term), soil organic matter, and sustainable N-cycling.

Options	SPECIFICATION REVISION
	There is no simple way for Caltrans to modify the specification.
	Develop a research program to better correlate the levels of soil N (short- to long-term), soil organic matter, and N-cycling with inoculated legume seeding rates to achieve target N-augmentation levels.

BULLETIN 1842
SEED INOCULATION AND FIELD PROBLEMS

7. Do not mix acid fertilizers with inoculated seeds, and do not sow seeds in contact with such fertilizers. The acidity may kill most or all of the root-nodule bacteria.

8. For the same reason, do not mix the seeds with fertilizers that contain trace elements, unless the manufacturer has specifically formulated and recommended the fertilizer product for that use.

9. Do not use herbicides, fungicides. or any other pesticides when planting inoculated seeds. Many of these poisons are highly toxic to root-nodule bacteria.

10. Check the acidity of the soil where inoculated seeds will be planted. Legumes usually fail to nodulate when the soil is more acid (lower pH) than pH 5.2. If the soil pH is too low. an appropriate amount of lime should be added to the soil at the time of planting. Drilling lime in with the seed is suitable, but coating seeds with calcium carbonate accomplishes the same thing and is compatible with broadcasting the seeds.

11. Make sure the soil contains adequate amounts of plant nutrients. A legume suffering from a deficiency of any nutrient other than nitrogen cannot benefit fully from inoculation. To maximize grazing potentials the legumes must have an adequate supply of available phosphorus and sulfur, the elements most commonly deficient on California rangelands. Many pasture legumes such as subterranean clover are less competitive than grasses for soil nutrients.

12. Do not use nitrogen fertilizers when planting legumes in a pasture. Well-nodulated legumes do not need any nitrogen from the soil, and nitrogenous fertilizers usually give grasses a competitive advantage over legumes. Moreover, ammonia, nitrates, and nitrites inhibit legume nodulation under most circumstances.

4.10 Site Physical Conditions

Many physical factors of the site strongly influence the success of effective nodulation and N_2-fixation [**104**]. Negative conditions include very high or low soil temperatures, low soil moisture, extremes of soil pH, soil fertility levels, chemical seed treatments, chemical fertilization, and the presence of indigenous or naturalized rhizobia can suppress legume nodulation by cultured inocula [**66**,**103**,**132**,**133**,**134**].

UC-AES Bulletin 1842 **outlines six instructional guidelines regarding site physical conditions (see sidebar). None of these factors are addressed directly in the Standard Specification.**

4.10.1 High Temperatures

High soil temperatures can negatively affect nodulation and N_2-fixation of legume crops [**105**]. Critical maximum temperatures for N_2-fixation in agricultural crops range from 30°C for clover and pea, 40°C for soybeans, guar, peanut and cowpeas [**120**]. Rhizobia are more susceptible to moist heat than dry heat [**160**,**174**]. Temperature affects root hair infection and nodule structure and function [**125**,**127**]. High but not extreme temps will delay root nodulation [**64**]. In field experiments, of the rhizobia that actually survive the inoculation and storage processes to be sown, 83% died after only 22.5h in the soil [**129**].

4.10.2 Nutrient Availability

Nodulation and N_2-fixation are inhibited by inorganic forms of N, mainly nitrate [**60**,**123**,**158**] and the presence of nitrate in soils has profound implications for the establishment of an effective symbiosis [**8**,**77**,**148**). Since soil nutrient availability is a primary factor driving this symbiosis, fertilization has significant effects upon the nature of the interaction. With adequate nitrogen levels in the soil following fertilization, nodulation is less likely to occur [**98**,**143**,**152**,**156**). Deficiencies in other nutrients such as phosphorous [**80**,**98**,**100**], molybdenum, and sulfur [**89**] can result in symbiosis failure.

4.10.3 Effects of Native or Naturalized Rhizobia

Root-nodule bacteria occur in the soil wherever legumes normally grow [**118**]. Soils almost invariably contain populations of rhizobia that may be indigenous, native to the area, and/or naturalized, persisting subsequent to introduction via agricultural or rangeland practices [**24**].

These rhizobia can have a major impact on the establishment and symbiotic performance of inoculant rhizobia [**147**]. Where there are large competitive populations of rhizobia in the soil inoculation is invariably futile [**24**,**97**].

While inoculation has been advantageous with agricultural crops, under conditions with native rhizobial populations it can be unnecessary. It has been demonstrated that indigenous rhizobia can both enhance plant establishment and increase soil fertility [**177**]. Under conditions where cultured strains of rhizobia may perish from suboptimal environmental factors such as low moisture or high temperatures, indigenous rhizobia are more likely to be well-suited to their native conditions [**66**,**86**] and, in theory, they could be more successful at effecting nodulation.

Rates of N_2-fixation for native Californian rhizobia are not well studied; many indigenous rhizobia may be highly competitive at nodulation but may not necessarily be efficient at N_2-fixation. Strains of rhizobia within a single species can interact with the host using one of three strategies: mutualistic (N_2-fixing for the host), non-symbiotic (unable to infect and nodulate with host), or parasitic (infect host but do not fix significant amounts of N_2) [**49**]. Data are lacking regarding roadside rhizobial densities before and after road construction activity in the state.

Problem 4.10.1 ► Information Standardization and Access

- **The information contained in *UC-AES Bulletin 1842* is not presented in an efficient format for individual Caltrans projects.**
- **Some of the guidelines have been ignored or overlooked as evidenced from individual project specifications [compiled in 34]; e.g., many projects require both inoculated legumes in the seed mix and nitrogen fertilization that will serve to suppress N_2-fixation.**
- **In addition, not all of the information presented in the Bulletin is written for the Caltrans context; the rancher/rangeland perspective does vary in many aspects from roadside revegetation.**

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

All appropriate staff should be issued copies of *UC-AES Bulletin 1842*, however, because it is not completely applicable to the Caltrans context, it should be condensed and reformatted to more briefly define and standardize the ancillary requirements necessary when legume inoculation is chosen as an N-augmentation option.

Problem 4.10.2 ► Lack of Site Soil Data

- **There are no requirements to quantify soil fertility or pH in order to ascertain the need for legume inoculation or chemical fertilization.**
- **There are no specifications regarding testing for indigenous rhizobia that may suppress the inoculated rhizobia. Landscape architects must make decisions regarding environmental factors in the absence of data.**

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

Landscape architects must be able to make N-augmentation decisions based on data. Each project should require a data acquisition protocol for the post-construction roadside including: soil fertility, soil pH, % organic matter, and the presence of indigenous or naturalized rhizobia.

Problem 4.10.3 ► Season of Seed Application

- **High and low soil temperatures can kill any cultured rhizobia that have survived the inoculation and seed application procedures. Desiccation in a dry seed bed can do the same.**
- ***UC-AES Bulletin 1842* recommends that inoculated seed be planted into a moist seedbed that will receive a germinating rain soon thereafter.**
- **There are no specifications restricting the seasonal timing of seed application. Seeding during the hot and dry months of late spring, summer, or early fall will likely kill all inoculant.**
- **The landscape contractor must comply with contractual time limits that are beyond the control of the seed specification designer and do not consider climatic or biological time constraints.**

Options

SPECIFICATION REVISION

There is no simple way for Caltrans to modify the specification.

The *UC-AES Bulletin 1842* should be adhered to with regard to timing of seed application upon a moist seedbed. Revegetation contracts with inoculated legumes should restrict seed application time frames to seasonal climatic window appropriate to the site.

Section 5
Actions & Recommendations

Action Alternatives

Recommended Alternative

5.1.1 Discontinue Or De-emphasize Current Legume Inoculation Practices.

Other Alternatives

5.1.2 Implement Basic Procedural Changes To Legume Seed Standard Specifications.

5.1.3 Implement Significant Procedural Changes To Standard Specifications.

5.1.4 Effect Research Regarding Legume Inoculation On Caltrans Roadsides Prior To Changing The Standard Specifications.

No Action

5.1.5 No Change : Do Not Change Legume Seed Standard Specifications.

This section provides several Action Alternatives regarding Standard Specification 20-2.10, and recommendations for routine soil fertility testing, topsoil stockpiling, and soil nitrogen augmentation options.

5.1 ACTION ALTERNATIVES

Following are five **Action Alternatives** regarding Standard Specification 20-2.10. **Table 5-1** is a summary of which legume inoculation factors are under control of manufacturers, of specifications, or of landscape contractors, and the potential modifications to improve efficacy.

Table 5-1. Summary of Present Control of Legume Inoculation Factors and Potential Modifications to Improve Efficacy.

		CONTROLLED BY				MODIFICATION POTENTIAL & TYPE
	LEGUME INOCULATION FACTORS	Inoculum Manufacturer	State Standard Specs	Project Specs	Landscape Contractor	1 Minor Modification to Specification; 2 Procedural Changes Beyond Specification; 3 Contracted Technical Research; 4 Product Quality and Compliance Testing
INOCULUM	Strain Selection	●	●			3
	Quality Control	●				4
	Storage Conditions	●				4
	Shelf Life	●	●			4
	Inoculation Provider		●		●	1
	Inoculation Rate		●	●		1
	Inoculation Method		●			1
	Post-Inoculation Storage Conditions		●		●	1
	Post-Inoculation Storage Time		●	●		1
SEED	Seed Mix Rate			●		1
	Seed Application Method			●		2
	Seed Application Timing			●		2
SITE	Site Soil Fertility			●		2
	Soil Organic Matter			●		2
	Indigenous Rhizobia			●		2

Recommended Alternative

The recommendation of this review is that Caltrans abandon SS 22-2.10 or limit its use to special cases at the discretion of project designers.

Recommended routine soil fertility testing, topsoil stockpiling, and other means for soil nitrogen augmentation, would likely provide greater long-term management benefits.

5.1.1 DISCONTINUE OR DE-EMPHASIZE CURRENT LEGUME INOCULATION PRACTICES

The existing practices make it unlikely that much, if any, N_2-fixation from cultured legume inoculants is occurring during roadside revegetation. The degree to which inoculated legume N_2-fixation has been effective on roadside revegetation projects remains undocumented. The possibility exists, though unlikely, that data demonstrating effectiveness of legume inoculation in a roadside context may be held by an inoculant manufacturer, seed vendor, or other party, but this review found no evidence that such data have been made public.

Comments:

There are critical procedures carried out by the manufacturer over which Caltrans has no control: selection of rhizobial strains, inoculant purity, and pre-sale inoculant storage conditions. Beyond these there are procedures that can be addressed by the Caltrans Standard Specifications which could improve the probability of successful N_2-fixation. However, these changes (**Action Alternatives 5.1.2** and **5.1.3**) may prove too cumbersome or costly for Caltrans to incorporate.

The following problems with present practices argue against the cost-effectiveness of continuing to require legume seed inoculation under SS 22-2.10:

1) the largely undocumented effectiveness of non-native cultured rhizobia at augmenting N_2-fixation for native legume species seeded by Caltrans;

2) the likely desiccation and death of rhizobia before, during, and after application of legume seed;

3) the typically low legume seeding rates (0.2-3 plants per ft^2) on most projects;

4) the very negative effects on rhizobia when inoculated seed is hydroapplied; and

5) the contravening use of commercial N fertilizer to promote rapid plant cover that inhibits rhizobial inoculation and nodulation.

Therefore, the recommendation of this review is that Caltrans abandon SS 22-2.10 or limit its use to special cases at the discretion of project designers. The following recommendations for routine soil fertility testing, topsoil stockpiling, and other means for soil nitrogen augmentation, would likely provide greater long-term management benefits.

5.1.1.1 Recommendations

5.1.1.1.1 Topsoil Stockpiling And Reapplication

The removal and return of topsoil is a well-established restoration technique [20]. The loss of original vegetation cover is less disruptive to the mineral nutrient supply than where original soils are lost and subsoils remain [20]. The establishment and rate of development of micro-organisms associated with plant and soil processes in disturbed soils are greatly influenced by the composition of the materials used in forming the planting medium and the availability of biodegradable organic matter [124]. The process of stockpiling and re-applying topsoil or duff to post-construction roadsides is one of the most effective ways to retain organic matter, nitrogen and nutrient cycling within the soil ecosystem. It alleviates the requirement to physically manipulate and amend subsoils to reduce bulk density, increase nutrient availability and mycorrhizal infectivity [13]. In temperate climates nutrients are least likely to be limited in long-established topsoils [20].

In most situations this process also returns a viable seedbank to the roadside. This seedbank commonly consists of whatever the context vegetation immediately adjacent to the roadside may harbor, which could include native species or undesirable weeds. A botanical survey of the roadsides and the immediately adjacent land prior to construction activity will produce a list of probable seedbank species and provide a baseline from which any additional seed mix species may be added if deemed prudent. A seedbank analysis of the topsoil or duff would be another means of predicting the post-construction roadside species composition.

A soil seed bank inventory and analysis can be a predictor of species composition on revegetating roadsides..

5.1.1.1.2 Soil Fertility Testing

Testing for soil fertility levels is a prerequisite for developing restoration protocols [**13**]. Only post-construction soil fertility data can provide the information necessary to make informed decisions about augmenting soil nitrogen and other plant nutrients in order to achieve revegetation goals.

Soil fertility data are necessary to make informed decision about augmenting soil nitrogen and other plant nutrients.

5.1.1.1.3 Soil Nitrogen Augmentation

There are options for augmenting low N levels, each with advantages and disadvantages (**Table 5-2**). Except for biological nitrogen fixation (BNF) through inoculated legumes, these topics represent subjects for investigation that go beyond the scope of this review, but that ultimately should be considered under the broad umbrella of roadside revegetation.

Table 5-2. Nitrogen Augmentation Methods For Unvegetated Roadside Soils.

	INORGANIC		ORGANIC							
TYPE	**FERTILIZER**		**ORGANIC MATTER**		**INOCULATED LEGUME SEED**		**NON-LEGUME PLANTS**		**MICROBIOTIC CRUST**	
Source			Duff/Topsoil Compost Mulch		Nitrogen Fixing *Rhizobia* in contact with legume seed		Actinorhizal Interactions *Alnus, Myrica,* Rhamnaceae, Rosaceae with Actinomycete *Frankia*		Bacteria, Cyanobacteria, Green and Brown Algae, Mosses, Lichens, Liverworts, Fungi	
Effect	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*
SHORT TERM EFFECTS (YEAR 1)	• immediate • slow-release available	• inorganic N leaches easily, especially in 1st rain event of year • high N levels may promote weeds & suppress native species	• immediate • organic N • inorganic N • soil biota present including free-living N_2 fixing bacteria & mycorrhizal fungi • seedbank	• seedbank of undesired species • must be affixed to steep slopes	• available 2-3 weeks after germination • rhizobia persist in soil • N release is slow • germinates successfully where vegetation density low	• unknown success rates in extra-agricultural systems	• comparably effective at N_2-fixation • includes > 160 spp of angiosperms w/in 6-7 orders • N release slow	• slow cover from seed • consists of mostly woody & some herbaceous perennials • not commercially available	• 3.6-24.1% cover in yr 1 • resists water/ wind erosion • binds soil particles • expedites revegetation • hydro-application option	• requires on-site collection and reapplication • not commercially available
LONG TERM EFFECTS (YEAR 2+)		• does not persist; reapply annually • vegetation failure if N levels drop below plant growth thresholds	• slow release • nutrient cycling	• seedbank of undesired species	• rhizobia persist in soil • rhizobia naturalize	• annuals short term only • unknown success rates in extra-agricultural systems	• long-lived	• slow cover from seed	• long-lived • cover increases rapidly for about 2 decades, then stabilizes	• sometimes slow to cover soil in arid climates

5.1.2 IMPLEMENT BASIC PROCEDURAL CHANGES TO LEGUME SEED STANDARD SPECIFICATIONS

Ascertain the quantitative need for inoculation. Require post-construction soil testing, especially fertility, for every project requiring revegetation (Section 3.10).

If N-augmentation is required, select the most appropriate method for short- or long-term options (see **Table 5-2**).

If specifying legume inoculation:

- Define inoculation rates for all species of legumes used in seed mix (Section 3.6, 4.6);
- Shorten the time between legume seed inoculation and seed application (Section 3.7, 4.7);
- Define post-inoculation seed storage conditions: refrigeration & transport in cooler to site (Section 3.7, 4.7);
- Restrict the timing of roadside seed application to coincide with impending rainfall season (Section 3.10, 4.10);
- Dry broadcast legume seeds separately; do not add them into the hydroseed mixture (Section 3.8, 4.8);
- Do not fertilize with N (Section 3.10, 4.10);
- Augment other nutrients critical to N_2-fixation as needed (Section 3.10, 4.10).

Reformat and condense ***UC-AES Bulletin 1842*** in order to define and standardize the practice of legume-inoculation as a method of N-augmentation.

Comments:

- Recognize that N-augmentation decisions should be data-driven.
- In situations with suboptimal N levels, legume germination failure or ineffective N_2-fixation could result in revegetation failure without N-augmentation via chemical fertilizers.

5.1.3 IMPLEMENT SIGNIFICANT PROCEDURAL CHANGES TO STANDARD SPECIFICATIONS

Implement Alternative 5.1.3 plus the following:

- Require an inoculant purity test (Section 3.4, 4.4) and viability test (Section 3.5, 4.5) for every project with legume inoculation;
- Require a seed inoculation test to quantify and confirm the average number of rhizobia per seed for each species of legume called for (Section 3.6, 4.6).

Although not strictly related to legume inoculation, implementation of a data acquisition agenda for every project would promote data-driven decisions regarding post-construction revegetation.

The following procedures would benefit every revegetation project:

- Test for soil organic matter (augment as needed);
- Test for soil seed bank if topsoil remains or is harvested and re-applied (augment seed application accordingly).

Comments:

Testing for inoculant purity, viability, and average number per seed should be the responsibility of the contractor, and documentation should be provided to verify compliance.

5.1.4 EFFECT RESEARCH REGARDING LEGUME INOCULATION ON CALTRANS ROADSIDES PRIOR TO CHANGING THE STANDARD SPECIFICATIONS

Quantify the success of legume inoculation as currently executed.

Has nodulation been successful?

If not, at which step could modifications be most effective?

Has N_2-fixation been successful for each legume species in the seed mix?

Quantify the effects of indigenous rhizobia.

Are they aiding or inhibiting N_2-fixation?

Quantify the amount of N input potential for all legume species used.

What are the rates of N_2-fixation for every species?

Develop guidelines to correlate legume seeding rates with target N input levels.

How many plants of each legume species are required per ft^2 to input a desired quantity of N?

Quantify the persistence of legumes on-site over 5 consecutive years.

Over what time span can N-augmentation be expected from legumes?

Explore other N-augmentation alternatives.

Would other alternatives be more effective for different regions of the State, e.g., microbiotic crusts for desert areas?

Comments:

Alternatives 5.1.2 and **5.1.3** contain recommendations based on a targeted literature review of the subject. The suggestions for research in **Alternative 5.1.4** address ways to assess the effectiveness of current practices and to make decisions based on empirical results.

5.1.5 NO CHANGE : Do not change legume seed standard specifications

Assumes that district level landscape architects can best ascertain:

1) the need for N-augmentation through the use of inoculated legumes;
2) appropriate project-specific modifications to the standard specifications or ***UC-AES Bulletin 1842***.

Comments:

- Recognize that landscape architects must make N-augmentation decisions in the absence of any soil fertility data.
- There are no data available that quantify the reliability and effectiveness of current practices of legume inoculation along Caltrans roadsides.
- There are no data available that quantify the amount of N-augmentation that could be expected from the current seeding rates of native legumes.

Section 6
References

1. **Aber JD, Nadelhoffer KJ, Steudler P, Melillo JM. 1989.** Nitrogen saturation in northern forest ecosystem. Bioscience 39:378-386.

2. **Allen EB. 1988.** Some trajectories for succession in Wyoming sagebrush grassland: implications for restoration. pp 89-112 ***In*** Allen EB, ed. The Reconstruction of Disturbed Arid Lands: an Ecological Approach. Westview Press: Boulder, CO.

3. **Allen EK, Allen ON. 1961.** The scope of nodulation in the Leguminosae. pp 585-588 ***In*** Recent Advances in Botany. Volume 1. Proceedings 9th International Botanical Congress. University of Toronto Press: Toronto, Canada.

4. **Ammann KAJ. 1999.** Significance of legumes and symbiotic N_2 fixation for grassland ecosystems along an altitudinal gradient in the Alps. PhD Dissertation, Swiss Federal Institute of Technology: Zurich, Switzerland.

5. **Baer SG, Blair JM, Collins SL, Knapp AK. 2003.** Soil resources in newly established tallgrass prairie. Ecology 84:724-735.

6. **Bashan Y, Hernandez J-P, Leyva LA, Bacilio M. 2002.** Alginate microbeads as inoculant carriers for plant-growth promoting bacteria. Biology and Fertility of Soils 35:359-368.

7. **Bell F, Nutman PS. 1971.** Experiments on nitrogen fixation by nodulated lucerne. Plant and Soil Special Volume:231-264.

8. **Bergersen FJ. 1982.** Root Nodules of Legumes: Structure and Functions. Research Studies Press, John Wiley and Sons Ltd: New York, NY.

9. **Bergersen FJ, Turner GL, Chase DL, Gault RR, Brockwell J. 1985.** The natural abundance of 15N in an irrigated soybean crop and its use for the calculation of nitrogen fixation. Australian Journal of Agricultural Research 36:411-423.

10. **Binkley D. 1983.** Ecosystem production in Douglas-fir plantations: interaction of red alder and site fertility. Forest Ecology and Management 5:215-227.

11. **Binkley D, Lousier JD, Cromack K. 1984.** Ecosystem effects of Sitka alder in a Douglas-fir plantation. Forest Service 30:26-35.

12. **Bloomfield HE, Handley JF, Bradshaw AD. 1982.** Nutrient deficiencies and the aftercare of reclaimed derelict land. Journal of Applied Ecology 19:151-158.

13. **Boerner REJ, Scherzer AJ, Brinkman JA. 1998.** Spatial patterns of inorganic N, P availability, and organic C in relation to soil disturbance: a chronosequence analysis. Applied Soil Ecology 7:159-177.

14. **Böhm M. 1992.** Air quality and deposition. pp 63-152 ***In*** Olson RK, Binkley D, Böhm M, eds. The Response of Western Forests to Air Pollution. Springer-Verlag: New York, NY.

15. **Bolton H Jr, Smith JL, Link SO. 1993.** Soil microbial biomass and activity of a disturbed and undisturbed shrub-steppe ecosystem. Soil Biology and Biochemistry 25:545-554.

16. **Boonkerd N, Weber DF, Bezdicek DF. 1978.** Influence of *Rhizobium japonicum* strains and inoculation methods on soybean grown in rhizobia-populated soils. Agronomy Journal 70:547-549.

17. **Boring LR, Swank WT. 1984.** The role of black locust (*Robinia pseudoacacia*) in forest succession. Journal of Ecology 72:749-766.

18. **Boring LR, Swank WT, Waide JB, Henderson GS. 1988.** Sources, fates, and impacts of nitrogen inputs to terrestrial ecosystems: review and synthesis. Biogeochemistry 6:119-159.

19. **Bradshaw AD. 1999.** The importance of nitrogen in the remediation of degraded land. pp 153-173 ***In*** Wong MH, Wong JWC, Baker AJM, eds. Remediation and Management of Degraded Lands. Lewis Publishers: Boca Raton, FL.

20. **Bradshaw AD. 2004.** The role of nutrients and the importance of function in the assembly of ecosystems. pp 325-340 ***In*** Temperton VM, Hobbs RJ, Nuttle T, Halle S, eds. Assembly Rules and Restoration Ecology: Bridging the Gap between Theory and Practice. Island Press: Washington, DC.

21. **Brockwell J. 1971.** An appraisal of an IBP experiment on nitrogen fixation by nodulated legumes. Plant and Soil Special Volume:265-272.

22. **Brockwell J, Bottomley PJ. 1995.** Recent advances in inoculant technology and prospects for the future. Soil Biology and Biochemistry 27:683-697.

23. **Brockwell J, Roughley RJ. 1967.** An examination of the numbers of nodule bacteria associated with legume seed following commercial multiple inoculation. Journal Australian Institute of Agricultural Science 33:204-207.

24. **Brockwell J, Bottomley PJ, Thies JE. 1995.** Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. Plant and Soil 174:143-180.

25. **Brockwell J, Roughley RJ, Herridge DF. 1987.** Population dynamics of *Rhizobium japonicum* strains used to inoculate three successive crops of soybean. Australian Journal of Agricultural Research 22:61-74.

26. **Brockwell J, Herridge DF, Roughley RJ, Thompson JA, Gault RR. 1975.** Studies on seed pelleting as an aid to legume seed inoculation: examination of pre-inoculated seed. Australian Journal of Experimental Agriculture and Animal Husbandry 15:780-787.

27. **Brown CS, Bugg RL. 2001.** Effects of established perennial grasses on the introduction of native forbs in California. Restoration Ecology 9:38-48.

28. **Brown MR, Wolf D, Morse RD, Neal JL. 1983.** Viability of *Rhizobium* in fertilizer slurries used for hydroseeding. Journal of Environmental Quality 12:388-390.

29. **Bytnerowicz A, Fenn ME. 1996.** Nitrogen deposition in California forests: a review. Environmental Pollution 92(2):127-146.

30. **Bytnerowicz A, Padgett PE, Parry SD, Fenn ME, Arbaugh MJ. 2001.** Concentrations, deposition, and effects of nitrogenous pollutants in selected California ecosystems. The Scientific World 1:304-311.

31. **Caldwell M. 1985.** Cold Deserts. pp 198-212 ***In*** Chabot BF, Mooney HA, eds. Physiological Ecology of North American Plant Communities. Chapman and Hall: Ne York, NY.

32. **Caldwell BE, Weber DF. 1970.** Distribution of *Rhizobium japonicum* serogroups in soybean nodules as affected by planting dates. Agronomy Journal 62:12-14.

33. **Catroux G, Hartman A, Ravellin C. 2001.** Trends in rhizobial inoculant production and use. Plant and Soil 230:21-30.

34. **CH2MHILL. 2002.** Statewide Erosion Control Review. Final Report CTS-RT-01-067. California Department of Transportation: Sacramento, CA.

35. **Chapin FS. 1980.** The mineral nutrition of wild plant. Annual Review of Ecology and Systematics 11:233-260.

36. **Chapman AG. 1935.** The effects of black locust on associated species with special reference to forest trees. Ecological Monographs 5:37-60.

37. **Claassen VP (Editor). 2005.** Soil Resource Evaluation. Final Report, California Department of Transportation: Sacramento, California.

38. **Claassen VP, Hogan MP. 2002.** Soil nutrient pools associated with revegetation of disturbed sites in the Lake Tahoe area. Restoration Ecology 10:195-203.

39. **Claassen VP, Zasoski RJ. 1998.** A comparison of plant available nutrients on decomposed granite cut slopes and adjacent natural soils. Land Degradation and Development 9:35-46.

40. **Connell JA, Sousa WP. 1983.** On the evidence needed to judge ecological stability or persistence. American Naturalist 121:789-824.

41. **Crews TE, Peoples MB. 2004.** Legumes versus fertilizer sources of nitrogen: ecological tradeoffs and human needs. Agriculture Ecosystems and Environment 102:279-297.

42. **da Silva PG, Bartolome JW. 1984.** Interaction between a shrub, *Baccharis pilularis* subsp. *consanguinea* (Asteraceae), and an annual grass, *Bromus mollis* (Poaceae), in coastal California. Madroño 31:93-101.

43. **Dancer WS, Handley JF, Bradshaw AD. 1977.** Nitrogen accumulation in kaolin mining wastes in Cornwall. II. Forage legumes. Plant and Soil 48:303-314.

44. **Date RA. 1977.** Inoculation of tropical pasture legumes. pp 293-311 ***In*** Vincent JS, Whitney AS, Bose J, eds. Exploiting the Legume-*Rhizobium* Symbiosis in Tropical Agriculture. University of Hawaii: Honolulu, Hawaii.

45. **Date RA, Roughley R. 1977.** Preparation of legume inoculants. pp 243-276 ***In*** Hardy RWF, Gibson AH, eds. A Treatise on Dinitrogen Fixation. John Wiley and Sons: Chichester, UK.

46. **Davidson EA, Stark JM, Firestone MK. 1990.** Microbial production and consumption of nitrate in an annual grassland. Ecology 7:1968-1975.

47. **Deaker R, Roughley RJ, Kennedy IR. 2004.** Legume seed inoculation technology—a review. Soil Biology and Biochemistry 36:1275-1288.

48. **DeGrood SH, Claassen VP, Scow KM. 2005.** Microbial community composition on native and drastically disturbed serpentine soils. Soil Biology and Biochemistry 37:1427-1435.

49. **Denison RF, Kiers KT. 2004.** Lifestyle alternatives for rhizobia: mutualism, parasitism and forgoing symbiosis. FEMS Microbiology Letters 237:187-193.

50. **Diatloff A, Langford S. 1975.** Effective natural nodulation of peanuts on Queensland. Journal of Agriculture and Animal Science 32:95-100.

51. **Dubach MM, Russelle MP. 1994.** Forage legume roots and nodules and their role in nitrogen transfer. Agronomy Journal 86:259-266.

52. **Dudman WF, Brockwell J. 1968.** Ecological studies of root-nodule bacteria introduced into field environments: I. A survey of field performance of clover inoculants by gel immune diffusion serology. Australian Journal of Agricultural Research 19:739-747.

53. **Edmundson GC. 1976.** Plant Materials Study. A Search for Drought-Tolerant Plant Materials for Erosion Control, Revegetation, and Landscaping along California Highways. Final Report. USDA-SCS LMPC-1. California Department of Transportation: Sacramento, CA.

54. **Eliason SA, Allen EB. 1997.** Exotic grass competition in suppressing native shrubland re-establishment. Restoration Ecology 5:245-255.

55. **Evans RD, Belnap J. 1999.** Long-term consequences of disturbance on nitrogen dynamics in an arid ecosystem. Ecology 80:150-160.

56. **Fenn ME, Poth MA. 1998.** Indicators of Nitrogen Status in California Forests. pp 123-129 ***In*** Bytnerowicz A, Arbaugh MJ, Schilling SL, eds. Proceedings of the International Symposium on Air Pollution and Climate Change Effects on Forest Ecosystems. USDA Forest Service PSW-GTR-166.

57. **Fenn ME, Poth MA, Bytnerowicz A, Sickman JO, Takemoto BK. 2003b.** Effects of ozone, nitrogen deposition, and other stressors on montane ecosystems in the Sierra Nevada. pp 111-155 ***In*** Bytnerowicz A, Arbaugh MJ, Alonso R, eds. Ozone Air Pollution in the Sierra Nevada: Distribution and Effects on Forests. Elsevier: Oxford, UK.

58. **Fenn ME, Baron JS, Allen EB, Rueth HM, Nydick KR, Geiser L, Bowman WD, Sickman JO, Meixner T, Johnson DW, Neitlich P. 2003a.** Ecological effects of nitrogen deposition in the western United States. BioScience 53:404-420.

59. **Freudenberger DO, Fish BE, Keeley JE. 1987.** Distribution and stability of grasslands in the Los Angeles basin. Bulletin of the Southern California Academy of Sciences 86:13-26.

60. **Gantry FH, Diem HG, Dommergues YR. 1982.** Effect of inoculation with Glomus mossae on nitrogen fixation by field grown soybeans. Plant and Soil 68:321-329.

61. **Gebhart DL, Call CA, Weaver RW. 1993.** Dinitrogen fixation and transfer in legume-crested wheatgrass mixtures. Journal of Range Management 46:431-435.

62. **George MJ, Clawson J, Menke J, Bartolome J. 1985.** Annual rangeland forage productivity. Rangelands 7:17-19.

63. **Gibson AH, Date RA, Ireland JA, Brockwell J. 1976.** A comparison of competitiveness and persistence amongst five strains of *Rhizobium trifolii*. Soil Biology and Biochemistry 8:395-401.

64. **Graham PH. 1992.** Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. Canadian Journal of Microbiology 38:475-484

65. **Graham PH. 2005.** Practices an issues in the inoculation of prairie legumes used in revegetation and restoration. Ecological Restoration 23:187-195.

66. **Graham PH, Tlusty B, Beyhaut E. 2004.** Inoculated legumes and revegetation/roadside plantings. Final Report . Minnesota Department of Transportation, St. Paul, MN.

67. **Gray J. 1983.** Nutrient use by evergreen and deciduous shrubs in southern California: 1. Community nutrient cycling and nutrient-use efficiency. Journal of Ecology 71:21-41.

68. **Guo Q. 2001.** Early post-fire succession in California chaparral: changes in diversity, density, cover and biomass. Ecological Research 16:471-485.

69. **Ham GE, Cardwell VB, Johnson HW. 1971.** Evaluation of *Rhizobium japonicum* inoculants in soils containing naturalized populations of rhizobia. Agronomy Journal 63:301-303.

70. **Hanes T. 1977.** Chaparral. pp 417-470 ***In*** Barbour MG, Major J, eds. Terrestrial Vegetation of California. John Wiley and Sons: New York, NY.

71. **Hansen PJ, Garten CT. 1992.** Deposition of H15NO3 vapour to white oak, red maple and loblolly pine foliage: experimental observations and a generalized model. New Phytologist 122:329-337.

72. **Hart SC, Firestone MK, Paul EA, Smith JL. 1993.** Flow and fate of soil nitrogen in an annual grassland and a young mixed conifer forest. Soil Biology and Biochemistry 25:431-442.

73. **Havlin JL, Beaton JD, Tisdale SL, Nelson WL. 1993.** Soil Fertility and Fertilizers. Prentice Hall: Upper Saddle River, NJ.

74. **Hawkes CV. 2003.** Nitrogen cycling mediated by biological soil crusts and arbuscular mycorrhizal fungi. Ecology 84:1553-1562.

75. **Heichel GH, Henjum K. 1991.** Dinitrogen fixation, nitrogen transfer and productivity of forage legume-grass communities. Crop Science 31:202-208.

76. **Helmers H, Bonner JF, Kelleher JM. 1955.** Soil fertility: a watershed management problem in the San Gabriel Mountains of southern California. Soil Science 80:189-197.

77. **Herridge DF, Roughey RJ, Brockwell J. 1984.** Effect of rhizobia and soil nitrate on establishment and functioning of the soybean symbiosis in the field. Australian Journal of Agricultural Research 35:149-161.

78. **Hirsch AM, Lum MR, Downie JA. 2001.** What makes the rhizobia-legume symbiosis so special? Plant Physiology 127:1484-1492.

79. **Holding AJ, Lowe JF. 1971.** Some effects of acidity and heavy metals on the *Rhizobium*-leguminous plant association. Plant and Soil Special Volume: 153-166.

80. **Holford ICR. 1997.** Soil phosphorous: its measurement and its uptake by plants. Australian Journal of Soil Resources 35:227-239.

81. **Holland AA, Street JE, Williams WA. 1969.** Range-legume inoculation and nitrogen fixation by root-nodule bacteria. University of California Agricultural Experiment Station Bulletin 842. Davis, CA.

82. **Holling CS. 1973.** Resiliance and stability of ecological systems. Annual Reviews of Ecology and Systematics 4:1-23.

83. **Ike AF, Stone EL. 1958.** Soil nitrogen accumulation under black locust. Soil Science Society of America Proceedings 22:346-349.

84. **Ireland JA, Vincent JM. 1968.** A quantitative study of competition for nodule formation. pp 85-93 ***In*** Transactions of the International Congress Soil Science, 9th. Volume 2. Angus & Robertson: Sydney, Australia.

85. **Jackson LE, Strauss RB, Firestone MK, Bartolome JW. 1988.** Plant and soil nitrogen dynamics in California annual grassland. Plant and Soil 110:9-17.

86. **Jacot KA, Luscher A, Nosberger J, Hartwig UA. 1999.** Symbiotic N_2 fixation of various legume species along an altitudinal gradient in the Swiss Alps. Soil Biology and Biochemistry 32:1043-1052.

87. **Jenkins MB, Virginia RA, Jarrell M. 1987.** Rhizobial ecology of the woody legume mesquite (*Prosopis glandulosa*) in the Sonoran desert. Applied and Environmental Microbiology 53:36-40.

88. **Jha PK, Nair S, Gopinanthan C, Babu CR. 1995.** Suitability of rhizobia inoculated wild legumes *Argyolobium flaccidum, Astragalus graveolens, Indigofera gangetica and Lespedeza stenocarpa* in providing a vegetational cover in an unreclaimed limestone quarry. Plant and Soil 177:139-149.

89. **Jones MB, Ruckman JE. 1973.** Long-term effects of phosphorous, sulfur and molybdenum on a subterranean clover pasture. Soil Science 115:343-348.

90. **Keeley JE, Keeley SC. 1984.** Post-fire recovery of California coastal sage scrub. American Midland Naturalist 111:105-117.

91. **Kendle AD, Bradshaw AD. 1992.** The role of soil nitrogen in the growth of trees on derelict land. Arboriculture Journal 16:103-122.

92. **Klemmedson JO. 1979.** Ecological importance of actinomycete-nodulated plants in the western United States. Botanical Gazette 140 (Supplement):S91-96.

93. **Lange RT. 1961.** Nodule bacteria associated with the indigenous Leguminosae of southwest Australia. Journal of General Microbiology 61:351-359.

94. **Lawrie AC. 1983.** Relationships among rhizobia from native Australian legumes. Applied and Environmental Microbiology 45:1822-1828.

95. **Ledgard SF, Steele KW. 1992.** Biological nitrogen fixation in mixed legume/grass pastures. Plant and Soil 141:137-153.

96. **Logan TJ. 1989.** Chemical degradation of soil. Advances in Soil Science 11:187-222.

97. **Lowther WL, Johnson DA, Rumbaugh MD. 1987.** Distribution and symbiotic effectiveness of *Rhizobium meliloti* in rangeland soils of the Intermountain West. Journal of Range Management 40:264-267.

98. **Lum MR, Hirsch AM. 2003.** Roots and their symbiotic microbes: strategies to obtain nitrogen and phosphorous in a nutrient-limited environment. Journal of Plant Growth Regulation 21:369-382.

99. **Lupwayi NZ, Olsen PE, Sande ES, Keyser HH, Collins MM, Singleton PW, Rice WA. 2000.** Inoculant quality and its evaluation. Field Crops Research 65:259-270.

100. **Marschner H. 1995.** Mineral Nutrition of Higher Plants. London Academic Press: London, UK.

101. **Marufu L, Karanja N, Ryder M. 1995.** Legume inoculant production and use in South Africa. Soil Biology and Biochemistry 27:735-738.

102. **Materon LA, Hagedorn C. 1984.** Responses of crimson clover to inoculation with genetically marked strains of *Rhizobium trifolii*. Communications Soil Science Plant Annals 15:33-47.

103. **McDermott TR, Graham PH, Ferrey ML. 1991.** Competitiveness of indigenous populations of *Bradyrhizobium japonicum* serocluster 123 as determined using root tip marking procedure in growth pouches. Plant and Soil 135:245-250.

104. **Metcalf SJ. 2005.** Symbiotic Nitrogen Fixation and Establishment of Six Montana Native Legume Species. M.S. Thesis, Montana State University: Bozeman, MT.

105. **Michiels J, Verreth C, Vanderleyden J. 1994.** Effects of temperature stress on bean nodulating *Rhizobium* strains. Applied and Environmental Microbiology 60:1206-1212.

106. **Miller RW, Donahue RL. 1990.** Soils: An Introduction to Soils and Plant Growth. Prentice-Hall, Inc: Englewood Cliffs, NJ

107. **Moenne-Loccoz Y, Dipankar S, Krause ES, Weaver RW. 1994.** Plasmid profiles of rhizobia used in inoculants and isolated from clover fields. Agronomy Journal 86:117-121.

108. **Montagnini F, Haines BL, Boring LR, Swank WT. 1986.** Nitrification potentials in early successional black locust and in mixed hardwood forest stands in the southern Appalachians, USA. Biogeochemistry 2: 197-210.

109. **Morris WF, Wood DM. 1989.** The role of lupine in succession on Mount St. Helens: facilitation or inhibition. Ecology 70:679-703.

110. **Mummey DL, Stahl PD, Buyer JS. 2002.** Soil microbiological properties 20 years after surface mine reclamation: spatial analysis of reclaimed and undisturbed sites. Soil Biology and Biochemistry 34:1717-1725.

111. **Munshower FF. 1994.** Practical Handbook of Disturbed Land Revegetation. CRC Press: Boca Raton, Florida.

112. **Nilsen ET, Schlesinger WH. 1981.** Phenology, productivity and nutrient accumulation in the post-fire chaparral shrub Lotus scoparius. Oecologia 50:217-224.

113. **Nussbaum S, von Ballmoos P, Gfeller H, Schlunegger UP, Fuhrer J, Rhodes D, Brunold C. 1993.** Incorporation of atmospheric 15NO2-nitrogen into free amino acids by Norway spruce, *Picea abies* (L.)Karst. Oecologia 94:408-414.

114. **O'Connell AM. 1986.** Effect of legume understory on decomposition and nutrient content of eucalypt forest litter. Plant and Soil 92:235-248.

115. **Olsen PE, Rice WA, Collins MM. 1994.** Biological contaminants in North American legume inoculants. Soil Biology and Biochemistry 27:699-701.

116. **Padgett PE, Allen EB, Bytnerowicz A, Minnich RA. 1999.** Changes in soil inorganic nitrogen as related to atmospheric nitrogenous pollutants in southern California. Atmospheric Environment 33:769-781.

117. **Peoples MB, Ladha JK, Herridge DF. 1995.** Enhancing legume N_2 fixation through plant and soil management. Plant and Soil 174:83-101.

118. **Phillips DA, Williams A. 1987.** Range-legume inoculation and nitrogen fixation by root-nodule bacteria. University of California Agricultural Experiment Station Bulletin 1842. Davis, CA.

119. **Philpotts H. 1977.** Survival of *Rhizobium trifolii* strains on inoculated seed held at 35° C. Australian Journal of Experimental Agriculture and Animal Husbandry 17:995-997.

120. **Piha ML, Munnus DN. 1987.** Sensitivity of the common bean (*Phaseolus vulgaris* L.) symbiosis to high soil temperature. Plant and Soil 98:183-194.

121. **Quackenbush FW, Carter AS, Shenberger LC. 1961.** Inspection of legume inoculants and growth substances. Inspection Report No. 28. Purdue University Agricultural Experiment Station: Lafayette, IN.

122. **Raven PH, Evert RF, Eichorn SE. 1992.** Biology of Plants. Fifth Edition. Worth Publishers: New York, NY.

123. **Redecker DP, Vonbereswordtwallrabe P, Beck DP, Werner D. 1997.** Influence of inoculation with arbuscular mycorrhizal fungi on stable isotopes of nitrogen in Phaseolus vulgaris. Biology and Fertility of Soils 24:344-346.

124. **Rothwell FM, Eagleston D. 1985.** Microbial relationships in surface-mine revegetation. Environmental Geochemistry and Health 7:28-35.

125. **Roughley RJ. 1970.** The influence of root temperature, *Rhizobium* strain and host selection on the structure and nitrogen-fixing efficiency of the root nodules of Trifolium subterraneum. Annals of Botany 34:631-646.

126. **Roughley RJ, Brockwell J. 1987.** Grain legumes and soil micro-organisms. pp 66-69 ***In*** De Kantzow DR, May MG, eds. Grain Legumes: Research and Production Seminar. AIAS Occasional Publication No. 28. Australian Institute of Agricultural Science: Sydney.

127. **Roughley RJ, Dart PJ. 1970.** Root temperature and root-hair infection of *Trifolium subterraneum* L. cv. Cranmore. Plant and Soil 32:518-520.

128. **Roughley RJ, Blowes WM, Herridge DF. 1976.** Nodulation of Trifolium subterraneum L. by introduced rhizobia in competition with naturalized strains. Soil Biology and Biochemistry 8:403-407.

129. **Roughley RJ, Gemell LG, Thompson JA, Brockwell J. 1993.** The number of *Bradyrhizobium* sp. (*Lupinus*) applied to seed and its effect on rhizosphere colonization, nodulation and yield of lupin. Soil Biology and Biochemistry 25:1453-1458.

130. **Schall ED, Schenberger L. Swope A. 1975.** Inspection of legume inoculants and pre-inoculated seeds. Inspection Report No. 106. Purdue University Agricultural Experiment Station: Lafayette, IN

131. **Schlesinger WH, Hasey MM. 1980.** The nutrient content of precipitation, dry fallout, and intercepted aerosols in the chaparral of southern California. American Midland Naturalist 103:114-122.

132. **Schwinning S, Parsons AJ. 1996a.** Analysis of the coexistence mechanisms for grasses and legumes in grazing systems. Journal of Ecology 84:799-813.

133. **Schwinning S, Parsons AJ. 1996b.** A spatially explicit population model of stoloniferous N-fixing legumes in mixed pasture with grass. Journal of Ecology 84:815-826.

134. **Schwinning S, Parsons AJ. 1999.** The stability of grazing systems revisited: spatial models and the role of heterogeneity. Functional Ecology 13:737-747.

135. **Shivaran S, Rai PV, Hedge SV. 1988.** Nitrogen fixation and nitrogen balance studies in *Rhizobium*-VA mycorrhiza inoculated legume-grass association by 15N isotope dilution technique under a field condition. Plant and Soil 111:11-16.

136. **Singleton PW, Tavares JW. 1986.** Inoculation response of legumes in relation to the number and effectiveness of indigenous *Rhizobium* populations. Applied and Environmental Microbiology 51:1013-1018.

137. **Singleton PW, Abdel-Magid HM, Tavares JW. 1985.** Effect of phosphorous on the effectiveness of strains of *Rhizobium japonicum*. Soil Science Society of America Journal 49:613-616.

138. **Skipper HD, Palmer JH, Giddens JE, Woodruff JM. 1980.** Evaluation of commercial soybean inoculants from South Carolina and Georgia. Agronomy Journal 72:673-674.

139. **Smith RS. 1992.** Legume inoculant formulation and application. Canadian Journal of Microbiology 38:485-492.

140. **Stephens JHG, Rask HM. 2000.** Inoculant production and formulation. Field Crops Research 65:249-258.

141. **Stevenson FJ. 1986.** Cycles of Soil: Carbon, nitrogen, phosphorous, sulfur, micronutrients. John Wiley & Sons: New York, N.Y.

142. **Stoddard JL. 1994.** Long-term changes in watershed retention of nitrogen: its causes and aquatic consequences. pp 223-284 ***In*** Baker, LA, ed. Environmental Chemistry of Lakes and Reservoirs. Advances in Chemistry Series No. 237. American Chemical Society: Washington, DC, USA.

143. **Streeter JG. 1998.** Inhibition of legume nodule formation and N_2 fixation by nitrate. Critical Reviews in Plant Sciences 7:1-22.

144. **Stylinski CD, Allen EB. 1999.** Lack of native species recovery following severe exotic disturbance in southern California shrublands. Journal of Applied Ecology 36:544-554.

145. **Suding KN, Gross KL, Houseman GR. 1984.** Alternate states and positive feedbacks in restoration ecology. Trends in Ecology and Evolution 19:46-53.

146. **Tarrant RF. 1983.** Nitrogen fixation in North American forestry: research and application. pp 261-278 ***In*** Gordon JC, Wheeler CT, eds. Biological Nitrogen Fixation in Forest Ecosystems: Foundations and Applications. Martinius Nijhoff/Dr. W. Junk Publishers: The Hague, Netherlands.

147. **Thies JE, Singleton PW, Bohlool BB. 1991a.** Modeling symbiotic performance of introduced rhizobia in the field by use of indices of indigenous population size and nitrogen status of the soil. Applied and Environmental Microbiology 57:29-37.

148. **Thies JE, Singleton PW, Bohlool BB. 1991b.** Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. Applied and Environmental Microbiology 57:19-28.

149. **Thompson JA. 1991.** Australian quality control standards. pp 107-111 ***In*** Thompson JA, ed. Report on the Expert Consultation on Legume Inoculant Production and Quality Control. FAO, United Nations: Rome.

150. **Trappe JB, Franklin JF, Tarrant RF, Hansen GM, eds. 1968.** Biology of alder. USDA Forest Service Pacific Northwest Forest and Range Experiment Station: Portland, OR.

151. **Turco RF, Kennedy AC, Jawson MD. 1994.** Microbial indicators of soil quality. pp 73-90 ***In*** Doran JW, Coleman DC, Bezdicek DF, Stewart BA, eds. Defining Soil Quality for a Sustainable Environment, SSSA Special Publication No. 35, Soil Science Society of America: Madison, WI.

152. **Unkovich M, Pate JS. 2000.** An appraisal of recent field measurements of symbiotic N_2 fixation by annual legumes. Field Crops Research 65:211-228.

153. **Vallentine JF. 1990.** Grazing Management. Academic Press:New York, NY.

154. **Van Kekeri L, Kay BL. 1986.** Revegetation of Disturbed Lland in California: an Element of Mined Land Reclamation. Open-file Report 86-14 SAC. California Department of Conservation, Division of Mines and Geology: Sacramento, California.

155. **van Rhijn P, Vanderleyden J. 1995.** The *Rhizobium*-plant symbiosis. Microbiological Reviews 59:124-142.

156. **Vance CP. 2001.** Symbiotic nitrogen fixation and phosphorous acquisition. Plant nutrition in a world of declining renewable resources. Plant Physiology 127:390-397.

157. **Vasek FC, Lund LL. 1980.** Soil characteristics associated with primary succession on a Mojave Desert dry lake plant community. Ecology 61:1013-1018.

158. **Vincent JM. 1965.** Environmental factors in the fixation of nitrogen by the legume. pp 384-435 ***In*** Bartholome WV, Clarke FE, eds. Soil Nitrogen, Agronomy Monograph 10, American Society of Agronomy: Madison, WI.

159. **Vincent JE, Smith MS. 1982.** Evaluation of inoculum viability on commercially inoculated legume seed. Agronomy Journal 74:921-922.

160. **Vincent JM, Thompson JA, Donovan KO. 1962.** Death of root-nodule bacteria on drying. Australian Journal of Agricultural Research 13:258-270.

161. **Vitousek P, Howarth RW. 1991.** Nitrogen limitation on land and in the sea: How can it occur? Biogeochemistry 13:87-115.

162. **Vitousek PM, Walker LR, Whiteacre LD, Mueller-Dombois D, Matson PA. 1987.** Bioinvasion by *Myrica faya* alters ecosystem development in Hawaii. Science 238:802-804.

163. **Vitousek PM, Cassman K, Cleveland C, Crews T, Field CB, Grimm NB, Howarth RW, Marino R, Martinelli L, Rastetter EB, Sprent JI. 2002.** Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry 57/58:1-45.

164. **Vose JM, Swank WT. 1990.** Preliminary estimates of foliar absorption of 15N-labeled nitric acid vapour (HNO3) by mature eastern white pine (Pinus strobus). Canadian Journal of Forest Resources 20:857-860.

165. **Wadoux P. 1991.** Inoculant production in industry using sterile carriers. pp 33-42 ***In*** Thompson JA, ed. Report on the Expert Consultation on Legume Inoculant Production and Quality Control. FAO, United Nations: Rome.

166. **Waldon, HB, Jenkins MB, Virginia RA, Harding EE. 1989.** Characteristics of woodland rhizobial populations from surface- and deep-soil environments of the Sonoran Desert. Applied and Environmental Microbiology 55:3058-3064.

167. **Wali MK. 1999.** Ecological succession and the rehabilitation of disturbed terrestrial ecosystems. Plant and Soil 213:195-220.

168. **Welker JM, Gordon DR, Rice KJ. 1991.** Capture and allocation of nitrogen by Quercus douglasii seedlings in competition with annual and perennial grasses. Oecologia 87:459-466.

169. **Wernergreen JJ, Harding EE, Riley MA. 1997.** *Rhizobium* gone native: unexpected plasmid stability of indigenous *Rhizobium leguminosarum*. Proceedings of the National Academy of Science 94:5483-5488.

170. **West NE, Klemmedson JO. 1978.** Structural distribution of nitrogen in desert ecosystems. pp 1-19 ***In*** West NE, Skujins J, eds. Nitrogen in Desert Ecosystems. US/IBP Synthesis Series Volume 9. Dowden, Hutchinson and Ross: Stroudsburg, PA.

171. **White DL. 1986.** Litter production, decomposition, and nitrogen dynamics in black locust and pine-hardwood stands of the southern Appalachians. M.S. Thesis. University of Georgia: Athens, GA.

172. **Whitford WG. 1986.** Pattern in desert ecosystems: water availability and nutrient interactions. pp 109-117 ***In*** Dubinsky Z, Steinberger Y, eds. Environmental Quality and Ecosystem Stability, Volume III. Bar Ilan University Press: Ramat Gan, Isreal.

173. **Whitford WG. 1988.** Decomposition and nutrient cycling in disturbed arid ecosystems. pp 136-161 ***In*** Allen EB, ed. The Reconstruction of Disturbed Arid Lands. An Ecological Approach. AAAS Selected Symposia Series 109. Westview Press: Boulder, CO.

174. **Wilkins J. 1967.** The effects of high temperature on certain root-nodule bacteria. Australian Journal of Agricultural Research 18:299-304.

175. **Zahran HH. 1998.** Structure of root nodules and nitrogen fixation in Egyptian wild herb legumes. Biol. Plant. 41:575-585.

176. **Zahran HH. 1999.** *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiology and Molecular Biology Reviews 63:968-989.

177. **Zahran HH. 2001.** Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation, and biotechnology. Journal of Biotechnology 91:143-153.

178. **Zink TA, Allen MF. 1998.** The effects of organic amendments on the restoration of a disturbed coastal sage scrub habitat. Restoration Ecology 6:52-58.

179. **Zink TA, Allen MF, Heindl-Tenhunen B, Allen EB. 1996.** The effect of a disturbance corridor on an ecological reserve. Restoration Ecology 3:304-310.

180. **Davis, CM. 1994.** Succession in California Shrub Communities Following Mechanical Anthopogenic Disturbance. MS Thesis, San Diego State University.

181. **Schellack, CH. 2000.** The Genetic Structure of *Rhizobium* Isolated From Natural Sierra Nevada Clover Populations. MS Thesis, Fresno State University.

182. **Tabatabai, F. 1985.** A Comparison of *Rhizobia* Infecting *Lotus purshianus*, and *Lupinus bicolor*. MS Thesis, Fresno State University.

183. **Villalobos, ML. 1994.** Determination of Diversity Host Association of *Rhizobium leguminosarum* biovar *trifolii* Through Plasmid Profile Analysis. MS Thesis, Fresno State University.

Appendix A
Glossary

Actinorhizal Symbioses. The N_2-fixing partnerships between actinomycete bacteria in the genus *Frankia* and a wide range of mostly woody angiosperms.

Ammonia Volatilization. When ammonium ($N0_3^-$) is converted from an organic to a gaseous form.

Anion. Negatively charged ion.

Anthropogenic. Human-caused or associated.

Atmospheric N Deposition. N compounds in the atmosphere that are returned to earth.

Bioavailable N. N in an inorganic form, nitrate or ammonium, that plants are able to absorb through their roots.

Biological N Fixation (BNF). The conversion of atmospheric nitrogen (N_2) directly into living organic tissues by specific prokaryotic organisms.

C:N. The ratio of %C to %N in organic residue, soil organic matter, and soil microorganisms; whether N is mineralized or immobilized depends on the C:N ratio of the organic matter being decomposed.

Cation. Positively charged ion.

Cross-Inoculation Group. A group of plant species amongst which a strain of rhizobia are freely interchangeable in terms of the ability to produce nodules.

Denitrification. Process that converts nitrate (NH4+) into nitrogen gas, mostly N_2.

Dinitrogen Fixation. See Nitrogen Fixation.

Duff. A product of litter decomposition; incompletely decomposed organic matter.

Effective Nodulation. When a rhizobium successfully nodulates with a legume.

Effective Rhizobia. When a rhizobium successfully nodulates and effects N_2-fixation with a legume.

Epiterranean Vegetation. Above-ground vegetation.

Facilitation. The successional process whereby a plant so modifies its environment that the environment becomes better suited for other plant species.

Free-Living. Non-symbiotic organisms.

Host. The legume component of the legume-rhizobia partnership.

Host Preference or Specificity. The degree to which a strain of rhizobia is able to effect nodule formation and N_2-fixation with a species of legume.

Immobilization. The conversion of an element from the inorganic to the organic form in microbial or plant tissues.

Indigenous. Applied to a species that occurs naturally in an area; native.

Inhibition. The successional process whereby a plant so modifies its environment that the environment excludes other plant species.

Leaching. The downward movement and drainage of minerals, or inorganic ions, in solution from the soil by percolating water.

Legume. Member of the vascular plant family Leguminosae, also called Fabacae after the broadbean genus *Faba*.

Legume Seed Inoculation. The process of introducing cultures of microorganisms, rhizobia, externally to the seeds of legumes.

Mediterranean-Type Climate. A climate with hot and dry summers, mild to cool and wet winters.

Microbiotic Crust. Living soil crusts found throughout the world especially in arid and semiarid regions; *aka* cryptogamic crusts, cryptobiotic crusts, microphytic crusts; these crusts are formed by living organisms and their by-products creating a surface crust of soil particles bound together by organic materials; organisms include cyanobacteria, green and brown algae, mosses, lichens, liverworts, fungi, bacteria.

Microsymbiont. In a symbiotic partnership, the microbiotic component such as bacteria or fungi.

Mineralization. The microbial decomposition of organic matter that releases ammonium ions in an inorganic form.

Monoculture. The growing of a single crop species or cultivar.

Mulch. A loose surface either natural or man-made, composed of organic or mineral materials, deposited on top of soil to protect soil and plant roots.

Mycorrhizae. A symbiotic relationship between fungi and plant roots, from which both benefit.

N-augmentation. Addition of inorganic or organic sources of nitrogen.

N-deficient Soil. Soils in which the available nitrogen is so low that the growth of plants is impaired.

N-transfer. The transfer of nitrogen that was obtained through legume-rhizobial N_2-fixation to non-legumes in proximity.

Nitrification. Microbial transformation via oxidation of ammonium cations to nitrate anions which can be used by plants (ammonium salts to nitrites to nitrates).

Nitrogen Cycle. The circulation and conversion of nitrogen-containing compounds among the atmosphere, the waters, the soils, and living organisms.

Nitrogen (N_2)-Fixation. Conversion of gaseous dinitrogen (N_2) in the air to organic nitrogenous forms by certain bacteria, algae, and actinomycetes.

Nitrogen Saturation. The point at which N retention capacity by biota and soil chemical fixation mechanisms is exceeded.

Nodulation. The process whereby rhizobia penetrate a legume root and provoke a response that results in the formation of a root-nodule that surrounds the rhizobia.

Nodules. Enlargements or swellings on the roots of legumes inhabited by symbiotic nitrogen-fixing bacteria.

Obligately Dependent. The survival of one organism dependent upon the presence of another organism.

Oxidation. A reaction in which atoms or molecules gain oxygen or lose hydrogen or electrons.

Parent Material. In soils: the unconsolidated, chemically weathered mineral from which the A and B horizons may have developed by pedogenic processes.

Physicochemical. Involving physical and chemical processes.

Plant Litter. An accumulation of dead plant remains on the soil surface.

Primary Succession. A succession initiated on a newly produced bare area.

Prokaryote. A cell lacking a membrane-bound nucleus and membrane-bound organelles; a bacterium.

Rate of Inoculation. Minimum number of rhizobial inoculants applied per seed.

Recruitment. The influx of new members into a population by reproduction or immigration.

Revegetation. The re-establishment of vegetation on denuded areas.

Rhizobia. The entire group of bacteria that are capable of forming symbiotic root-nodule partnerships with legumes.

Rhizosphere. That part of the soil which is modified physically and chemically by the presence of plant roots.

Root-nodule Bacteria. See Rhizobia.

Senesce. The processes of deterioration that terminate naturally the life of an organism.

Soil Horizons. Developmentally-related layers of soil, each with a characteristic physical, chemical, and biological attribute.

Soil Microbial Biomass. The total mass of the living microorganisms in the soil.

Soil Microbiota. Bacteria, fungi, actinomycetes that reside in the soil.

Soil Organic Matter. Organic materials in all stages of decomposition.

Soil Seedbank. The ungerminated but viable seeds that lie in the soil.

Subsoil. The subsurface soil.

Succession. An ecological term referring to an orderly progression of changes in community structure and function.

Symbiosis. Intimate association of two dissimilar organisms; legume plants and bacteria, rhizobia, share a mutually beneficial symbiotic relationship.

Topsoil. The surface layer of soil containing organic matter, usually corresponding to the A horizon.

Trophic. A level in the transfer of food or energy within a chain.

Viable Bacteria. Having the capacity to live, grow, develop.